

# Cytokines Downregulate the Sarcoendoplasmic Reticulum Pump $\text{Ca}^{2+}$ ATPase 2b and Deplete Endoplasmic Reticulum $\text{Ca}^{2+}$ , Leading to Induction of Endoplasmic Reticulum Stress in Pancreatic $\beta$ -Cells

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Cytokines and free radicals are mediators of  $\beta$ -cell death in type 1 diabetes. Under in vitro conditions, interleukin-1 $\beta$  (IL-1 $\beta$ ) +  $\gamma$ -interferon (IFN- $\gamma$ ) induce nitric oxide (NO) production and apoptosis in rodent and human pancreatic  $\beta$ -cells. We have previously shown, by microarray analysis of primary  $\beta$ -cells, that IL-1 $\beta$  + IFN- $\gamma$  decrease expression of the mRNA encoding for the sarcoendoplasmic reticulum pump  $\text{Ca}^{2+}$  ATPase 2b (SERCA2b) while inducing expression of the endoplasmic reticulum stress-related and proapoptotic gene CHOP (C/EBP [CCAAT/enhancer binding protein] homologous protein). In the present study we show that cytokine-induced apoptosis and necrosis in primary rat  $\beta$ -cells and INS-1E cells largely depends on NO production. IL-1 $\beta$  + IFN- $\gamma$ , via NO synthesis, markedly decreased SERCA2b protein expression and depleted ER  $\text{Ca}^{2+}$  stores. Of note,  $\beta$ -cells showed marked sensitivity to apoptosis induced by SERCA blockers, as compared with fibroblasts. Cytokine-induced ER  $\text{Ca}^{2+}$  depletion was paralleled by an NO-dependent induction of CHOP protein and activation of diverse components of the ER stress response, including activation of inositol-requiring ER-to-nucleus signal kinase 1 $\alpha$  (IRE1 $\alpha$ ) and PRK (RNA-dependent protein kinase)-like ER kinase (PERK)/activating transcription factor 4 (ATF4), but not ATF6. In contrast, the ER stress-inducing agent

thapsigargin triggered these four pathways in parallel. In conclusion, our results suggest that the IL-1 $\beta$  + IFN- $\gamma$ -induced decrease in SERCA2b expression, with subsequent depletion of ER  $\text{Ca}^{2+}$  and activation of the ER stress pathway, is a potential contributory mechanism to  $\beta$ -cell death. *Diabetes* 54:452–461, 2005

The endoplasmic reticulum (ER) is a highly dynamic organelle, responsible for modification and sorting of newly synthesized proteins and  $\text{Ca}^{2+}$  storage and signaling (1). The resting free  $\text{Ca}^{2+}$  concentration in the ER is three to four orders of magnitude higher than cytosolic  $\text{Ca}^{2+}$ . This gradient is generated by sarcoendoplasmic reticulum  $\text{Ca}^{2+}$  ATPase (SERCA) proteins that pump  $\text{Ca}^{2+}$  into the ER and by Ins(1,4,5) $\text{P}_3$  and ryanodine receptors that release  $\text{Ca}^{2+}$  from the organelle (1). Disruption of ER homeostasis, as caused by alterations in ER  $\text{Ca}^{2+}$  concentration, leads to the accumulation of unfolded proteins and activation of a specific stress response (2). The ER stress response involves translational attenuation, an increase in the folding capacity of the ER by upregulation of ER chaperones, a degradation of misfolded proteins, and, in extreme cases, apoptosis. This is achieved by the coordinate transcription of mRNAs encoding several ER resident proteins via activation of inositol-requiring ER-to-nucleus signal kinase 1 $\alpha$  (IRE1 $\alpha$ ) and activating transcription factor 6 (ATF6) pathways. IRE1 $\alpha$  and ATF6 are transmembrane proteins that are cleaved from the ER membrane and translocate to the nucleus during ER stress. Cleaved ATF6 binds to the promoter of genes encoding ER chaperone proteins, increasing protein folding activity in the ER (2). Activation of IRE1 $\alpha$  results in the processing of mRNA encoding for another transcription factor, X-box binding protein-1 (xbp-1). This leads to the synthesis of an active xbp-1 protein, which also induces the expression of ER chaperone genes (3). In parallel, ER stress activates PRK (RNA-dependent protein kinase)-like ER kinase (PERK), which phosphorylates eukaryotic translation initiation factor 2 $\alpha$  (eIF2 $\alpha$ ), resulting in a downregulation of protein synthesis and the activation of transcription factor ATF4 (4). Under prolonged or excessive ER stress, elements of the apoptotic machinery are induced, including transcriptional activa-

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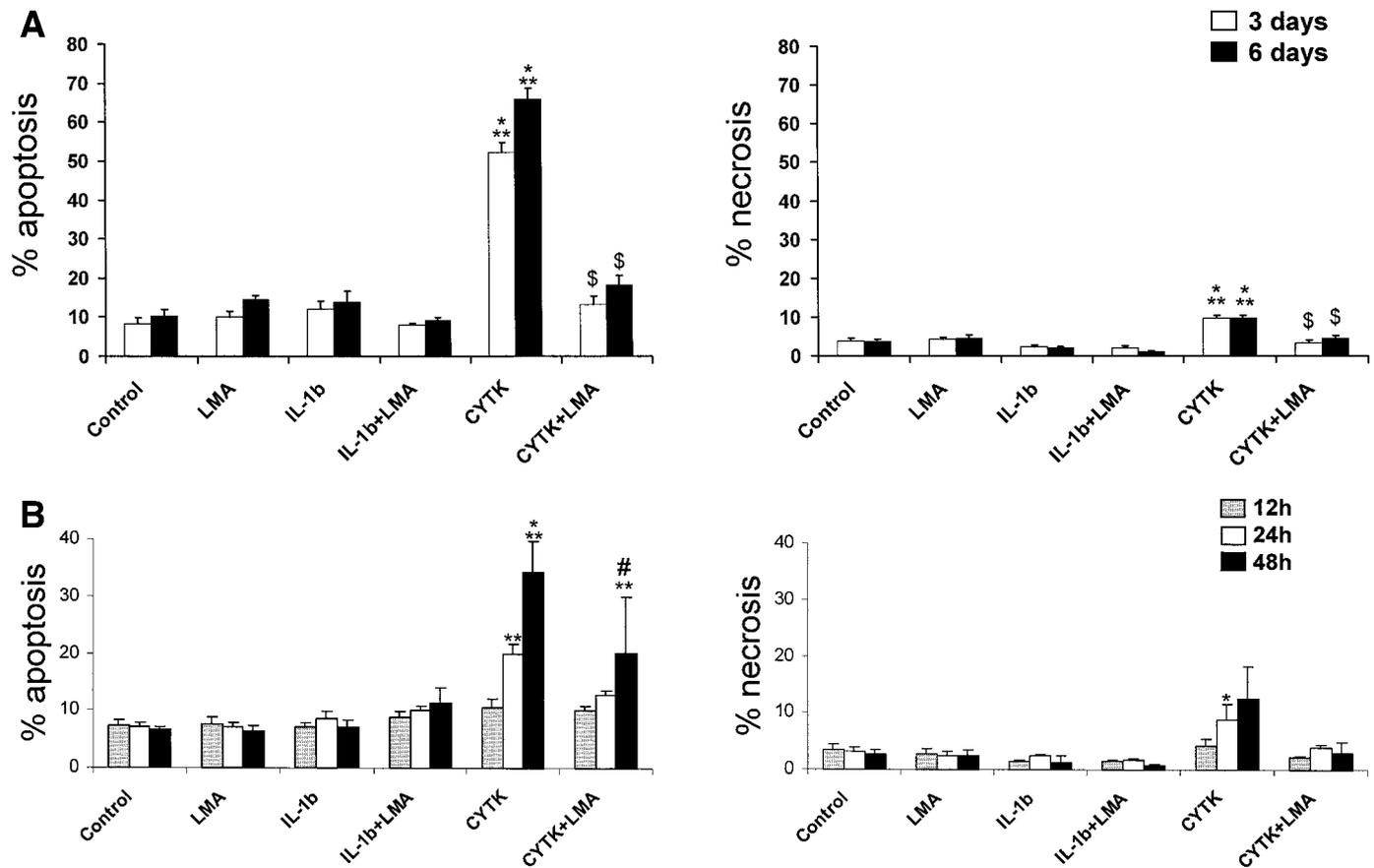
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ATF, activating transcription factor; BiP, immunoglobulin heavy-chain binding protein;  $[\text{Ca}^{2+}]_i$ , intracellular  $\text{Ca}^{2+}$  concentration; CHOP, C/EBP (CCAAT/enhancer binding protein) homologous protein; CPA, cyclopiazonic acid; eIF2 $\alpha$ , eukaryotic translation initiation factor 2 $\alpha$ ; ER, endoplasmic reticulum; FACS, fluorescence-activated cell sorting; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IFN- $\gamma$ ,  $\gamma$ -interferon; IL, interleukin; iNOS, inducible nitric oxide synthase; IRE1 $\alpha$ , inositol-requiring ER-to-nucleus signal kinase 1 $\alpha$ ; JNK, c-Jun NH<sub>2</sub>-terminal kinase; LMA, N<sup>G</sup>-methyl-L-arginine; PERK, PRK (RNA-dependent protein kinase)-like ER kinase; SERCA, sarcoendoplasmic reticulum  $\text{Ca}^{2+}$  ATPase; xbp-1, X-box binding protein-1.

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**FIG. 1.** Cytokine-induced cell death in primary rat  $\beta$ -cells and INS-1E cells is mostly NO dependent. **A:** Rat  $\beta$ -cells were exposed for 3 and 6 days to IL-1 $\beta$  (50 units/ml), IL-1 $\beta$  + IFN- $\gamma$  (cytokines [CYTK] 0.036  $\mu$ g/ml), or cytokines with or without the iNOS blocker LMA (1 mmol/l) or left untreated (control). **B:** INS-1E cells were treated for 12, 24, and 48 h as in 1A (except for the use of 10 units/ml IL-1 $\beta$ ). Apoptosis and necrosis are expressed as a percentage of the total number of cells counted. Primary  $\beta$ -cells:  $n = 4-7$ ; INS-1E cells:  $n = 5-15$ . \* $P \leq 0.05$ , \*\* $P \leq 0.01$ , \*\*\* $P \leq 0.001$  vs. control; # $P \leq 0.05$ , \$ $P \leq 0.001$  vs. cytokines, ANOVA.

tion of C/EBP (CCAAT/enhancer binding protein) homologous protein (CHOP; also called GADD153 [growth arrest-and DNA damage-inducible gene 153]) (5), IRE1 $\alpha$ -mediated caspase-12 cleavage, and activation of the c-Jun NH<sub>2</sub>-terminal kinase (JNK) pathway, leading to apoptosis (2,6,7).

IRE1 $\alpha$  and PERK are highly expressed in pancreatic  $\beta$ -cells (8,9). PERK moderates translation, thus limiting the load placed on the ER of secretory cells, and PERK knockout mice have progressive loss of  $\beta$ -cells and diabetes (9). This suggests that  $\beta$ -cells, because of their very active secretory characteristics, are particularly vulnerable to ER stress.

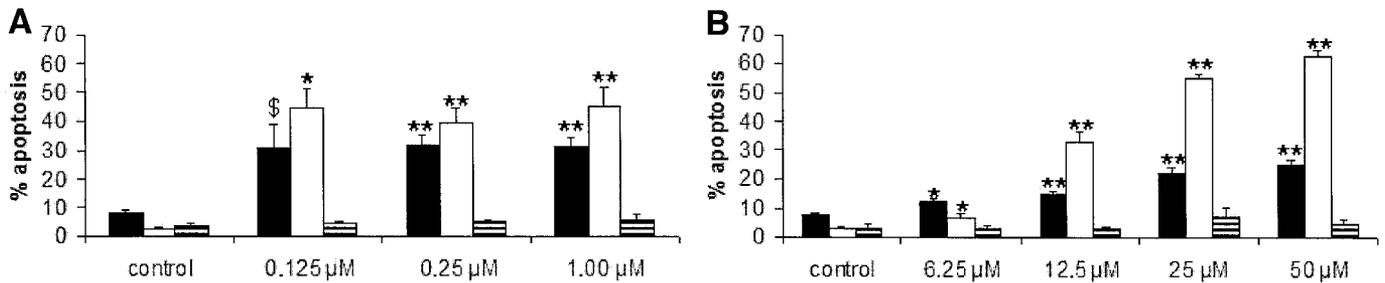
Type 1 cytokines, such as interleukin-1  $\beta$  (IL-1 $\beta$ ), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), and  $\gamma$ -interferon (IFN- $\gamma$ ), are mediators of  $\beta$ -cell death in type 1 diabetes (10). Under in vitro conditions, IL-1 $\beta$ , in combination with IFN- $\gamma$ , induces NO production, severe functional suppression, and death by apoptosis of pancreatic islet cells (10). Chemical NO donors deplete ER Ca<sup>2+</sup> in insulin-producing cells by an unknown mechanism, leading to ER stress, CHOP expression, and apoptosis (8). Of note, cytokine-induced  $\beta$ -cell death can, under some circumstances, be dissociated from NO production (11-13). Thus, although there is increasing evidence for an important contribution of ER stress in  $\beta$ -cell apoptosis, the role for ER stress in cytokine-mediated  $\beta$ -cell death and the molecular mechanisms mediating

cytokine-induced ER stress pathways in  $\beta$ -cells remain to be clarified.

We have previously observed by microarray analysis that IL-1 $\beta$  + IFN- $\gamma$  induce CHOP mRNA expression in primary  $\beta$ -cells and INS-1E cells, whereas it decreases expression of mRNA for the ER Ca<sup>2+</sup> pump SERCA2b (14-16). Departing from these observations, we have currently used fluorescence-activated cell sorting (FACS)-purified primary rat  $\beta$ -cells and INS-1E cells to clarify the different cytokine-induced ER stress pathways activated in these cells. The studies with cytokines were paralleled by observations with the SERCA inhibitor thapsigargin, a well-known ER stress-inducing agent previously shown to induce apoptosis in insulin-producing MIN6 and BRIN cells (17,18).

## RESEARCH DESIGN AND METHODS

Pancreatic islets were isolated from adult Wistar rats by collagenase digestion and  $\beta$ -cells purified by FACS (FACStar; Becton Dickinson, Sunnyvale, CA) (19). The preparations contained 90  $\pm$  2%  $\beta$ -cells ( $n = 6$ ). Purified  $\beta$ -cells were precultured for 16-40 h in Ham's F-10 medium (20). Because of difficulties in isolating large numbers of primary  $\beta$ -cells, experiments requiring >1 million cells were performed with the well-differentiated INS-1E cells (21). Cytokines induce apoptosis and a similar pattern of gene expression in INS-1E cells as compared with primary  $\beta$ -cells (15,16). INS-1E cells were cultured in RPMI medium (15). In some experiments  $\beta$ -cells were compared with rat fibroblasts (208F; ECACC, Salisbury, U.K.), cultured in Dulbecco's modified Eagle's medium supplemented with 10% FCS. The following cytokines were used: recombinant human IL-1 $\beta$  (a kind gift from Dr. C.W. Reynolds, National Cancer



**FIG. 2.**  $\beta$ -Cells are particularly susceptible to SERCA inhibition. Rat  $\beta$ -cells (■), INS-1E cells (□), and rat fibroblasts (▨) were exposed for 24 h to the SERCA blockers thapsigargin (A) or CPA (B) or left untreated (control). Apoptosis is expressed as a percentage of the total number of cells counted ( $n = 3-4$ ).  $\$P \leq 0.05$ ,  $*P \leq 0.02$ ,  $**P \leq 0.01$  vs. control,  $t$  test.

Institute, Bethesda, MD), at 50 units/ml for  $\beta$ -cells and 10 units/ml for INS-1E cells; and recombinant rat IFN- $\gamma$  (R&D Systems, Oxon, U.K.), at 0.036  $\mu$ g/ml for both  $\beta$ -cells and INS-1E cells (this concentration of IFN- $\gamma$  corresponds to  $\sim 100$  units/ml of recombinant mouse IFN- $\gamma$ ). The choice of cytokine concentrations used is based on our previous dose-response experiments (10,15,22 and B. Kutlu and D.L.E., unpublished data). The inducible NO synthase (iNOS) blocker  $N^G$ -methyl-L-arginine (LMA; Sigma, Steinheim, Germany) was used at 1.0 mmol/l, a concentration that prevents cytokine-induced NO production (23). The SERCA blockers thapsigargin and cyclopiazonic acid (CPA; Sigma, Steinheim, Germany) were used at different concentrations, as indicated in RESULTS. Culture media were collected at different time points for nitrite determination (nitrite is a stable product of NO oxidation) (24).

**Assessment of cell viability.** FACS-purified rat  $\beta$ -cells (10,000 cells/condition), INS-1E cells (6,000 cells/condition), and rat fibroblasts (6,000 cells/condition) were cultured attached to 96-well dishes. Primary  $\beta$ -cells and INS-1E cells were exposed to IL-1 $\beta$ , IFN- $\gamma$ , and LMA alone or in combinations for 3 and 6 days or 12, 24, and 48 h, respectively. FACS-purified rat  $\beta$ -cells, INS-1E cells, and fibroblasts were exposed to the SERCA blockers thapsigargin or CPA for 24 h. The percentage of viable, necrotic, and apoptotic cells were determined by inverted fluorescence microscopy with the DNA dyes Hoechst 342 (20  $\mu$ g/ml) and propidium iodide (10  $\mu$ g/ml) (11,15). Viability was evaluated by at least two independent observers (A.K.C. and F.O.), with one of them unaware of sample identity. The agreement between the findings obtained by the two observers was always  $>90\%$ .

**Intracellular  $Ca^{2+}$  concentration measurements.** FACS-purified  $\beta$ -cells (20,000 cells per condition) were plated on round glass coverslips. After preculture, cells were incubated for 24 h with cytokines and/or LMA or thapsigargin. Measurements of intracellular  $Ca^{2+}$  concentration ( $[Ca^{2+}]_i$ ) were performed using fura-2 acetoxymethyl ester as previously described (18,25).

Furaptra loading, cell permeabilization, and measurements of intracellular  $Ca^{2+}$  stores were performed as described (26), with some modifications (18). Free  $Ca^{2+}$  was buffered at 200 nmol/l with 2 mmol/l EGTA and the appropriate amount of  $Ca^{2+}$ , using the Max Chelator program (Stanford University). Calibration of furaptra was carried out as previously described (26), except that  $R_{max}$  and  $R_{min}$  were obtained in the presence of 4  $\mu$ mol/l digitonin and 10  $\mu$ mol/l ionomycin, either at saturating  $Ca^{2+}$  concentrations or in the absence of  $Ca^{2+}$ .

**Real-time RT-PCR analysis and evaluation of xbp-1 processing.** After different culture conditions, cells were harvested, Poly(A)<sup>+</sup> RNA was isolated using a Dynabeads mRNA direct kit (Dyna, Oslo, Norway), and an RT reaction was performed (16). Expression of SERCA2b, CHOP, immunoglobulin heavy-chain binding protein (BiP), and ATF4 mRNAs were determined by real-time RT-PCR using SYBR Green fluorescence on a LightCycler instrument (Roche Diagnosis, Mannheim, Germany) using the standard curve method (27,28). The

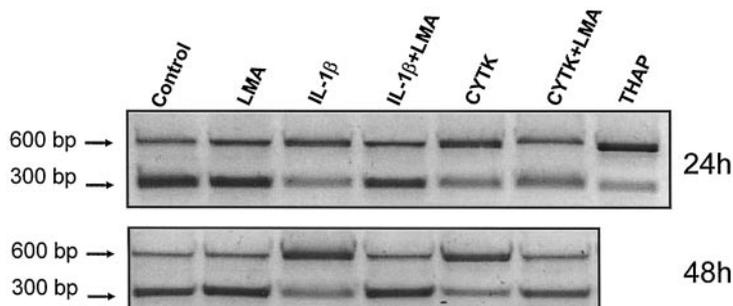
housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used for confirmation of similar cDNA loading. We have previously shown (16) and confirmed in the present series of experiments (data not shown) that cytokines do not modify GAPDH expression. Results are shown as the expression of the gene of interest divided by GAPDH and multiplied by a factor 10 for clarity. The PCR amplification reactions and preparation of standards were performed as previously described (27). Primers for real-time RT-PCR were: ATF4 forward: GTTGGTCAGTGCCTCAGACA, reverse: CATT CGAAACAGAGCATCGA (109 bp); CHOP: forward: CCAGCAGAGGTCA-CAAGCAC, reverse: CGCACTGACCACTCTGTTTC (127 bp); SERCA2b: forward: TTTGTGGCCCGAAACTACCT, reverse: GGCATAATGAGCAGCACA AAGGG (121 bp); GAPDH: forward: AGTTCAACGGCAGTCAAG, reverse: TACTCAGCACCAGCATCACC (118 bp); and BiP: forward: CCACCAGGATGC AGACATTG, reverse: AGGGCCTCCACTTCCATAGA (100 bp).

IRE-1 $\alpha$  activation leads to cleavage of xbp-1 mRNA. xbp-1 processing is characterized by excision of a 26-bp sequence from the coding region of xbp-1 mRNA (3). The cleaved fragment contains a Pst-1 restriction site, and the extent of xbp-1 processing can be thus evaluated by restriction analysis. A fragment of 601 bp of xbp-1 cDNA, encompassing the 26-bp excised region, was amplified by conventional PCR (29). PCR products were then purified and incubated with PstI restriction enzyme for 5 h at 37°C. Restriction digests were separated in 2% agarose gels containing ethidium bromide. PCR products derived from nonspliced xbp-1 mRNA (indicating absence of ER stress) were digested in two bands of  $\sim 300$  bp. In contrast, products amplified from spliced xbp-1 mRNA were resistant to digestion and remained 601 bp long, indicating presence of ER stress.

**Western blot analysis.** INS-1E cells were exposed to cytokines and thapsigargin for different time periods. An equal amount of protein was subjected to an 8–10% SDS-PAGE and transferred to a nitrocellulose membrane. Immunoblot analyses were performed with anti-CHOP (Sigma, Saint Louis, MO), anti-phospho JNK, anti-total JNK (Cell Signaling, Beverly, MA), and anti-SERCA2 (Santa Cruz Biotechnology, Santa Cruz, CA).

**ATF6 reporter assay.** A reporter plasmid containing the luciferase gene under the control of five ATF6 binding sites (30) was kindly provided by Prof. Prywes (Columbia University, NY). Of note, this construct might also be activated by xbp-1 under some experimental conditions (31). Luciferase activities were assayed with the Dual-Luciferase Reporter assay system (Promega, Madison, WI), as previously described (32,33). Test values were corrected for the luciferase value of the internal control plasmid pRL-CMV.

**Statistical analysis.** Data are shown as the means  $\pm$  SE, and comparisons between groups were made either by  $t$  test (paired or unpaired) or by ANOVA followed by  $t$  test with the Bonferroni correction or Tukey's post test, as indicated.  $P \leq 0.05$  was considered statistically significant.



**FIG. 3.** Cytokines induce xbp-1 processing in primary rat  $\beta$ -cells via NO production. Rat  $\beta$ -cells were exposed for 24 or 48 h to IL-1 $\beta$  (50 units/ml) or IL-1 $\beta$  + IFN- $\gamma$  (cytokines [CYTK], 0.036  $\mu$ g/ml), with or without LMA (1 mmol/l), or left untreated (control). Thapsigargin (THAP; 1  $\mu$ mol/l) was used as a positive control for ER stress. mRNA was extracted and cDNA utilized as a template for PCR using xbp-1-specific primers. After amplification, PCR products were incubated with the restriction enzyme Pst-1. PCR products derived from spliced xbp1 are left intact (600 bp), indicating the presence of ER stress. The figure shown is representative of four (24 h) and two (48 h) experiments.

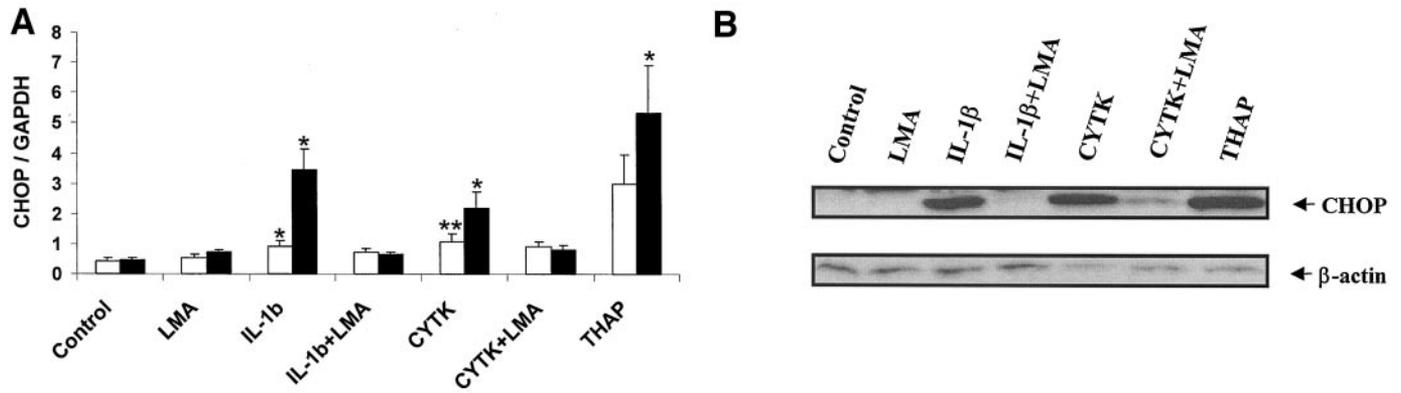


FIG. 4. Cytokine-induced CHOP mRNA and protein expression in  $\beta$ -cells is mediated by NO production. **A:** Rat  $\beta$ -cells were cultured for 6 h (□) or 24 h (■) in the presence of IL-1 $\beta$  (50 units/ml) or IL-1 $\beta$  + IFN- $\gamma$  (cytokines [CYTK] 0.036  $\mu$ g/ml), with or without LMA (1 mmol/l), or left untreated (control). Thapsigargin (THAP; 1  $\mu$ mol/l) was used as a positive control. mRNA was extracted and real-time RT-PCR performed, with correction for GAPDH. The results are the means  $\pm$  SE of 4–7 experiments. \* $P \leq 0.05$ , \*\* $P \leq 0.01$ ,  $t$  test. **B:** INS-1E cells were treated for 24 h as in **A** (except for the use of 10 units/ml IL-1 $\beta$ ) and then harvested for protein extraction. Sixty micrograms of total protein were immunoblotted using an anti-CHOP antibody and, as a housekeeping protein,  $\beta$ -actin. The figure is representative of three independent experiments.

## RESULTS

**IL-1 $\beta$  + IFN- $\gamma$  induce apoptosis of INS-1E cells and primary  $\beta$ -cells mainly via NO formation.** We first examined the role of NO for IL-1 $\beta$  + IFN- $\gamma$ -induced cell death. Neither IL-1 $\beta$  nor IFN- $\gamma$  alone induced cell death in rat  $\beta$ -cells (Fig. 1A) (IFN- $\gamma$  data not shown). When used in combination, IL-1 $\beta$  + IFN- $\gamma$  induced  $\beta$ -cell death mostly via apoptosis, with a minor necrotic component (Fig. 1A). Prevention of NO production by LMA abolished cytokine-induced necrosis and significantly decreased apoptosis in

primary cells (Fig. 1A). The levels of apoptosis, however, did not return to control levels ( $10 \pm 2\%$  after 6 days for control, as compared with  $18 \pm 2\%$  for cytokines + LMA). Cytokine-induced cell death is usually detected earlier in INS-1E cells than in primary  $\beta$ -cells (15). In line with this, a 24- to 48-h exposure to IL-1 $\beta$  + IFN- $\gamma$  induced a significant increase in INS-1E cell death, mainly by apoptosis (Fig. 1B). Blocking NO production prevented IL-1 $\beta$  + IFN- $\gamma$ -induced apoptosis after 24 h but had only a partial protection after 48 h, suggesting the presence of

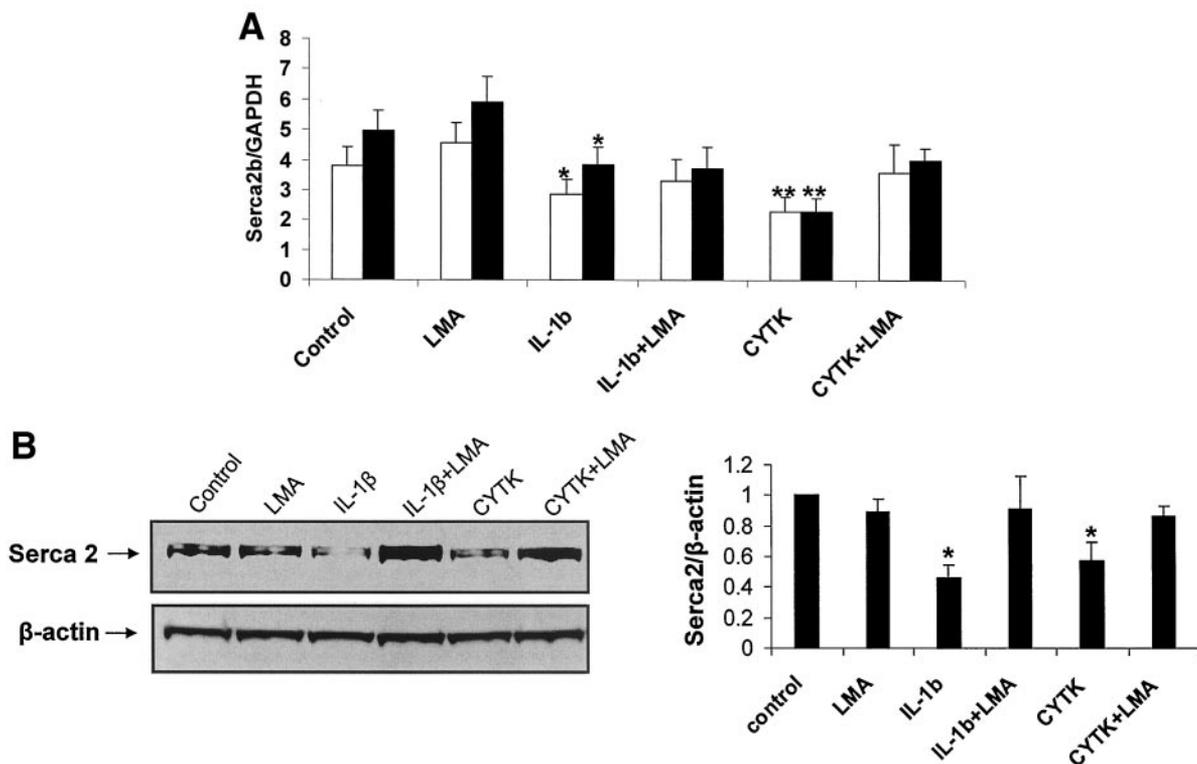
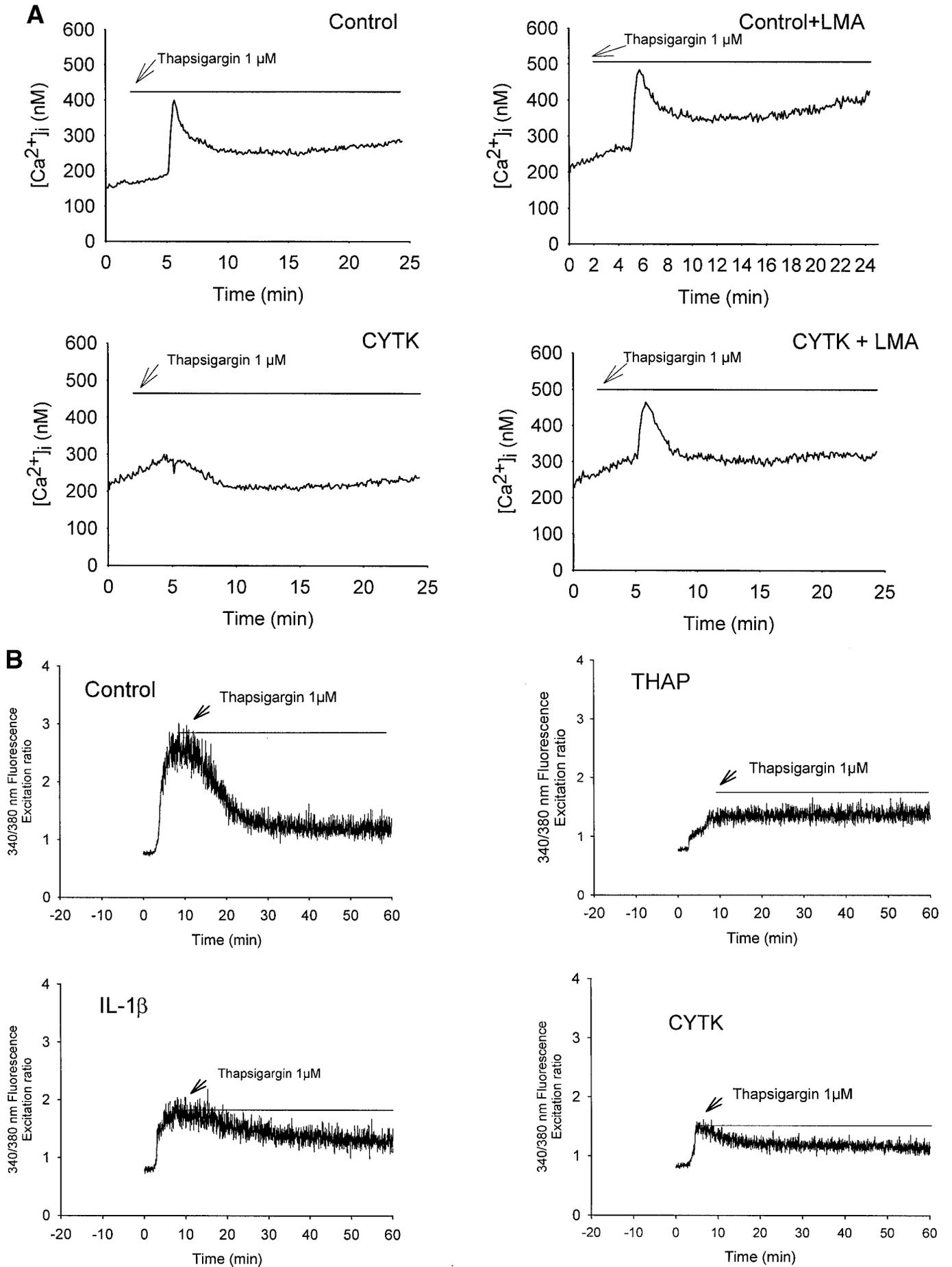
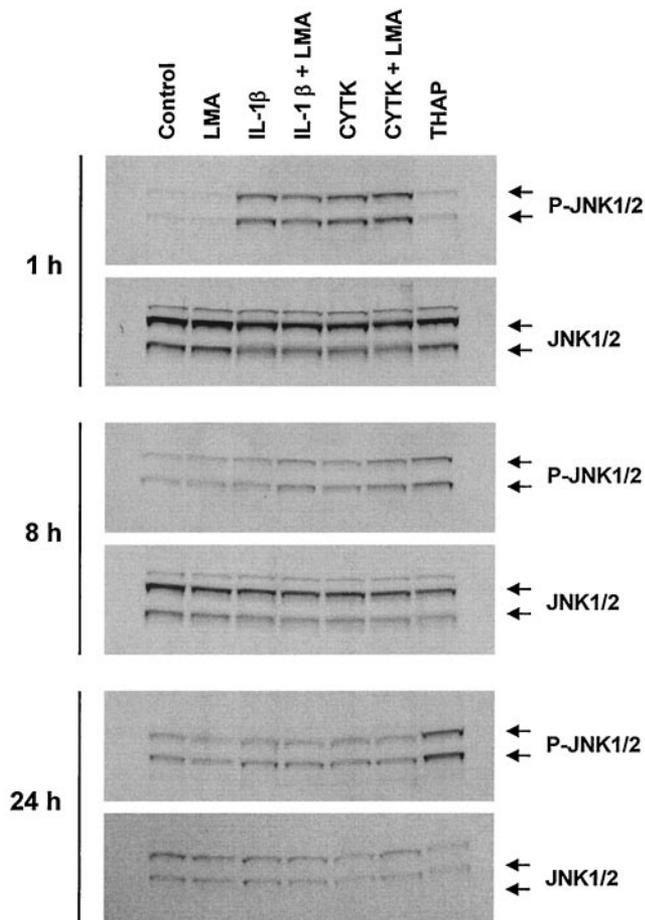


FIG. 5. Cytokines decrease SERCA2b mRNA and protein expression in  $\beta$ -cells. **A:** Rat  $\beta$ -cells were cultured for 6 h (□) or 24 h (■) as described in Fig. 4A. mRNA was extracted and real-time RT-PCR performed with correction for GAPDH expression. The results are the means  $\pm$  SE of 4–7 experiments. **B:** INS-1E cells were treated for 24 h as in Fig. 4B and then harvested for protein extraction. Twenty-five micrograms of total protein were immunoblotted using an anti-SERCA2 antibody and, as a housekeeping protein,  $\beta$ -actin. The graphic shows fold induction of SERCA2 corrected by  $\beta$ -actin as compared with control values. Data are the means  $\pm$  SE of five experiments. \* $P \leq 0.05$ , \*\* $P \leq 0.005$  vs. control condition, paired  $t$  test. CYTK, cytokines.





**FIG. 7.** Cytokines induce an early and transient activation of JNK in INS-1E cells. INS-1E cells were treated for 1, 8, and 24 h as described in Fig. 4B. Then, 20  $\mu$ g of total protein extracts were immunoblotted using antibodies against phosphorylated or total JNK 1/2. The experiment shown is representative of four independent experiments. CYTK, cytokines; THAP, thapsigargin.

additional and non-NO-mediated apoptotic signals. On the other hand, LMA completely prevented the minor necrotic component of cell death. Neither IL-1 $\beta$  (Fig. 1B) nor IFN- $\gamma$  alone (data not shown) induced INS-1E cell death. In line with our previous observations (14,15,23), LMA prevented cytokine-induced NO formation (data not shown). As positive controls for ER stress induction, cells were exposed to different concentrations of the SERCA blockers thapsigargin or CPA. Exposure of  $\beta$ -cells and INS-1E cells for 24 h to thapsigargin or CPA induced cell death mainly via apoptosis (Fig. 2), with a minor necrotic component (data not shown). Although 24-h exposure to thapsigargin induced apoptosis in both primary rat  $\beta$ -cells and INS-1E cells (Fig. 2A), there was no increase in fibroblast cell death (Fig. 2A); fibroblasts were only significantly killed after 48 h of thapsigargin exposure ( $47 \pm 2\%$  at  $1 \mu\text{mol/l}$ ). Similarly, although a 24-h exposure to  $25 \mu\text{mol/l}$  CPA induced  $22 \pm 2\%$  and  $55 \pm 1\%$  apoptosis in rat  $\beta$ -cells and INS-1E cells, respectively (Fig. 2B),  $250 \mu\text{mol/l}$  of CPA was necessary to induce  $23 \pm 3\%$  apoptosis in fibroblasts. Thus,

both primary  $\beta$ -cells and INS-1E cells are markedly susceptible to apoptosis induced by agents that deplete ER  $\text{Ca}^{2+}$ . The iNOS-blocking agent LMA failed to prevent thapsigargin or CPA-induced  $\beta$ -cell or INS-1E cell death (data not shown).

**IL-1 $\beta$  + IFN- $\gamma$  activates the ER stress response in primary  $\beta$ -cells.** To verify whether IL-1 $\beta$  or IL-1 $\beta$  + IFN- $\gamma$  activate the ER stress response in  $\beta$ -cells, we analyzed IRE-1 $\alpha$ -mediated splicing of xbp-1 mRNA in primary rat  $\beta$ -cells. As a positive control, cells were treated with thapsigargin. Most of the xbp-1 product was not cleaved by Pst-1 in the thapsigargin-treated cells, leading to a higher prevalence of the 600-bp nondigested product (Fig. 3), thus confirming the induction of ER stress. Similar results were observed with cells exposed to  $25 \mu\text{mol/l}$  CPA (data not shown). On the other hand, in both nontreated cells or cells treated with LMA alone, a large proportion of xbp-1 was cleaved (300-bp band) (Fig. 3), as expected from non-ER-stressed cells. Exposure of primary  $\beta$ -cells to IL-1 $\beta$  or IL-1 $\beta$  + IFN- $\gamma$ , but not to IFN- $\gamma$  alone (data not shown), led to an increased proportion of noncleaved xbp-1 (600 bp) (Fig. 3) after both 24 and 48 h. This pattern was prevented by LMA, suggesting that the cytokine-induced ER stress in  $\beta$ -cells is mostly mediated via NO formation. At 6 h, except for thapsigargin-treated cells, no difference in xbp-1 mRNA processing was observed (data not shown). LMA did not prevent thapsigargin- or CPA-induced xbp-1 splicing (data not shown).

CHOP was expressed at low levels in control  $\beta$ -cells, but expression of both mRNA and protein increased several-fold after exposure to IL-1 $\beta$ , IL-1 $\beta$  + IFN- $\gamma$ , or thapsigargin (Fig. 4A and B). This effect of cytokines is mostly mediated via NO formation, since it was decreased by LMA. LMA did not prevent thapsigargin-induced CHOP expression in primary  $\beta$ -cells or INS-1E cells (data not shown). There was no early (6 h) induction of CHOP protein by cytokines, and IFN- $\gamma$  alone did not induce CHOP expression (data not shown). Taken together, the cleavage of xbp-1 and the upregulation of CHOP suggest that cytokines induce an ER stress response in pancreatic  $\beta$ -cells.

**Cytokines downregulate SERCA2b mRNA and deplete ER calcium in primary  $\beta$ -cells via NO formation.** SERCA2b mRNA was decreased by 40–55% in  $\beta$ -cells exposed for, respectively, 6 or 24 h to a combination of IL-1 $\beta$  + IFN- $\gamma$ , an effect prevented by LMA (Fig. 5A). IL-1 $\beta$  also decreased SERCA2b mRNA, whereas IFN- $\gamma$  alone had no effect (data not shown). In line with the mRNA data, IL-1 $\beta$  or IL-1 $\beta$  + IFN- $\gamma$  decreased SERCA2 protein expression, an effect partially prevented by the iNOS blocker (Fig. 5B).

We next evaluated the effects of cytokines or thapsigargin on cytosolic  $\text{Ca}^{2+}$  ( $[\text{Ca}^{2+}]_i$ ) and ER  $\text{Ca}^{2+}$  concentrations. Thapsigargin induced a biphasic increase in  $[\text{Ca}^{2+}]_i$ , with an initial peak followed by a plateau phase (Fig. 6A, upper left panel). This increase results from the leak-out of  $\text{Ca}^{2+}$  from the ER in the presence of SERCA inhibition, and hence it indirectly reflects ER  $\text{Ca}^{2+}$  stores (18). In

**FIG. 6.** Cytokines deplete ER  $\text{Ca}^{2+}$  in primary  $\beta$ -cells. Rat  $\beta$ -cells were exposed for 24 h to IL-1 $\beta$  (50 units/ml) or IL-1 $\beta$  + IFN- $\gamma$  (cytokines [CYTK],  $0.036 \mu\text{g/ml}$ ), with or without LMA (1 mmol/l), or left untreated (control). Prolonged (24 h) preexposure to thapsigargin (THAP;  $1 \mu\text{mol/l}$ ) was used as a positive control for depletion of ER  $\text{Ca}^{2+}$ . A: Effect of thapsigargin ( $1 \mu\text{mol/l}$ , added 2 min onwards) on  $[\text{Ca}^{2+}]_i$  in primary  $\beta$ -cells. The traces shown are the means of three experiments ( $\sim 90$ – $135$  cells/experiment). B: Furaptra 340-nm-to-380-nm fluorescence excitation ratio in primary  $\beta$ -cells exposed to cytokines. The figures are representative of three similar experiments.

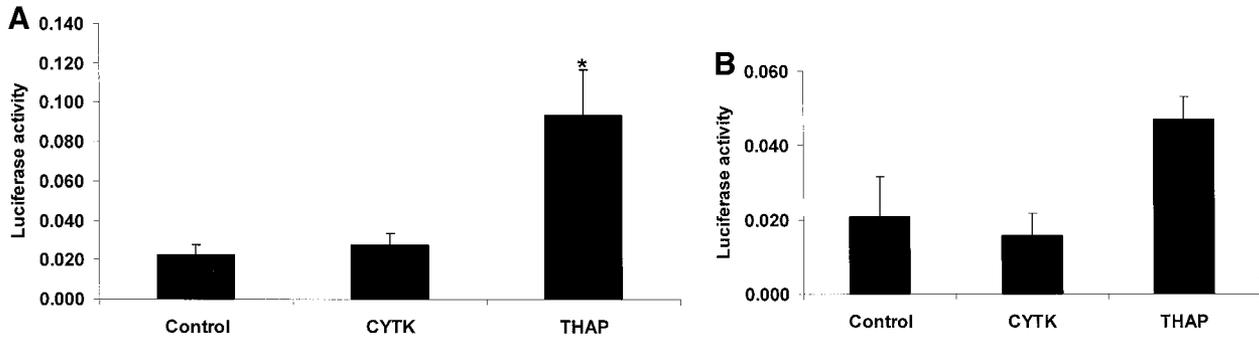


FIG. 8. Thapsigargin, but not cytokines, activate the ATF6 reporter gene in  $\beta$ -cells and INS-1E cells. INS-1E cells (A) and rat  $\beta$ -cells (B) were cotransfected with the 5xATF6 site luciferase reporter gene (p5xATF6GL3) and the internal control pRL-CMV, encoding *Renilla* luciferase. After overnight transfection, the cells were exposed for 24 h to IL-1 $\beta$  + IFN- $\gamma$  (cytokines [CYTK]) or thapsigargin (THAP) and assayed for firefly and *Renilla* luciferase activities. The results were normalized for *Renilla* luciferase activity ( $n = 3-4$ ). \* $P \leq 0.05$  vs. control,  $t$  test.

agreement with such a view, the acute exposure to thapsigargin failed to increase  $[Ca^{2+}]_i$  in  $\beta$ -cells cultured for 24 h in the presence of the SERCA inhibitor (data not shown). Preculture of  $\beta$ -cells for 24 h in the presence of IL-1 $\beta$  + IFN- $\gamma$  reduced the increase in  $[Ca^{2+}]_i$  induced by the acute exposure to thapsigargin ( $P \leq 0.01$ ,  $n = 3$ ) (Fig. 6A, lower left panel), indicating that exposure to the combination of cytokines depleted ER  $Ca^{2+}$  stores. This effect of IL-1 $\beta$  + IFN- $\gamma$  was partially prevented by LMA

(Fig. 6A, lower right panel). The low-affinity  $Ca^{2+}$  indicator fura2/AM was used to monitor free  $Ca^{2+}$  concentration in the ER of individual  $\beta$ -cells after controlled permeabilization of the plasma membrane (18,26). After recording the fluorescence obtained by excitation at 340 and 380 nm, cells were permeabilized in "intracellular medium" containing 4  $\mu$ M digitonin and 200 nmol/l  $Ca^{2+}$ . Upon the sudden drop in fluorescence caused by the loss of cytoplasmic fura2/AM, the detergent was removed while con-

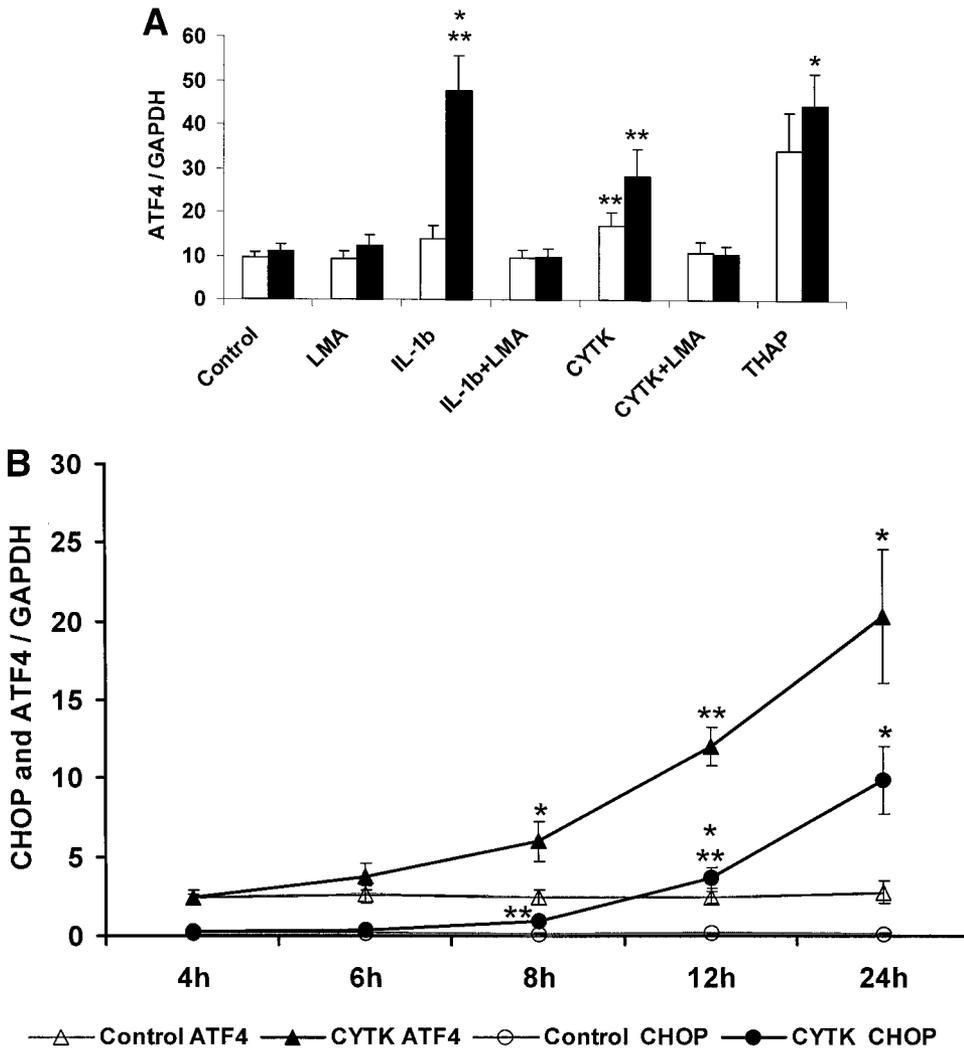


FIG. 9. A: Cytokines induce ATF4 mRNA expression in primary  $\beta$ -cells and INS-1E cells via NO formation. A: Rat  $\beta$ -cells were treated for 6 h ( $\square$ ) or 24 h ( $\blacksquare$ ) as described in Fig. 4A. mRNA was extracted and real-time RT-PCR performed with correction for GAPDH expression. The results are the means  $\pm$  SE of 4-7 experiments. B: INS-1E cells were exposed for different time points to IL-1 $\beta$  (10 units/ml) + IFN- $\gamma$  (cytokines [CYTK]; 0.036  $\mu$ g/ml) with or without LMA (1 mmol/l) (not shown) or left untreated (control). mRNA was extracted and real-time RT-PCR performed for ATF4 and CHOP with correction for GAPDH. The results are the means  $\pm$  SE of 4-5 experiments. \* $P \leq 0.05$ , \*\* $P \leq 0.01$ , \*\*\* $P \leq 0.005$  vs. control condition,  $t$  test. THAP, thapsigargin.

tinuing the measurement of the fluorescence at both wavelengths. The loss of cytoplasmic furaptra was associated with an inversion of the 340-nm-to-380-nm fluorescence excitation ratio, indicating that the remaining indicator was exposed to the higher concentrations of free  $\text{Ca}^{2+}$  prevailing in intracellular stores (18,26). This corresponds to the initial increase in fluorescence excitation ratio illustrated in Fig. 6B (upper left panel). This initial rise was reduced by  $53 \pm 7$  and  $68 \pm 6\%$  in IL-1 $\beta$ - and IL-1 $\beta$  + IFN- $\gamma$ -treated cells, respectively ( $P \leq 0.05$ ,  $n = 3$ ) (Fig. 6B, lower panels). When the fluorescence ratio is converted into  $\text{Ca}^{2+}$  concentrations, the initial increase in control cells was 212  $\mu\text{mol/l}$ . This peak was reduced to 54 and 32  $\mu\text{mol/l}$  in IL-1 $\beta$ - and IL-1 $\beta$  + IFN- $\gamma$ -treated cells, respectively (one representative experiment). We have previously observed that thapsigargin released 50% of the  $\text{Ca}^{2+}$  pool sensed by furaptra under the present condition (18). Therefore, thapsigargin was used to estimate  $\text{Ca}^{2+}$  stores in the ER. In control cells, the acute exposure to thapsigargin induced a  $\sim 80\%$  decrease in the furaptra 340-nm-to-380-nm fluorescence excitation ratio (Fig. 6B, upper left panel). This effect of thapsigargin was reduced by  $63 \pm 13$  and  $83 \pm 6\%$  in IL-1 $\beta$ - and IL-1 $\beta$  + IFN- $\gamma$ -treated cells, respectively (Fig. 6B, lower panels), compared with control cells ( $P \leq 0.05$  and  $P \leq 0.01$ , respectively;  $n = 3$ ). Again, the effect of IL-1 $\beta$  + IFN- $\gamma$  was partially prevented by LMA (data not shown). In cells cultured for 24 h in the presence of the SERCA inhibitor, thapsigargin failed to reduce the fluorescence excitation ratio (Fig. 6B, upper right panel). These functional data are in good agreement with the SERCA2b mRNA and protein observations, and they suggest that cytokine-induced NO formation leads to decreased SERCA2b expression and severe depletion of ER  $\text{Ca}^{2+}$ .

**Characterization of the ER stress pathways activated by cytokines in  $\beta$ -cells.** The data described above indicate that cytokines induce IRE-1 $\alpha$  activation/xbp-1 processing (Fig. 3). IRE-1 $\alpha$  activation activates the JNK pathway in fibroblasts (7). We thus determined phosphorylation of JNK 1/2 in INS-1E cells exposed to cytokines or thapsigargin. IL-1 $\beta$  or IL-1 $\beta$  + IFN- $\gamma$  induced an early (1 h) and marked JNK activation; JNK activity remained slightly increased after 8 h but returned to control levels after 24 h of continuous cytokine exposure (Fig. 7). In contrast, thapsigargin increased JNK activity only after 8 h, with peak activation after 24 h (Fig. 7). Thus, cytokines induce an early and transitory JNK activation in INS-1E cells that precedes both NO production and ER stress.

To characterize whether cytokines induce activation of ATF6 in  $\beta$ -cells, we used a construct containing the luciferase reporter gene downstream of five ATF6 binding sites (5xATF6). Although thapsigargin and CPA (data not shown) activated the ATF6 luciferase construct in INS-1E cells (Fig. 8A) and primary  $\beta$ -cells (Fig. 8B), a combination of cytokines failed to increase activity of the ATF6 construct in these cells. One of the known targets of ATF6 is the ER chaperone BiP. In agreement with the ATF6 promoter data,  $\beta$ -cells exposed for 24 h to IL-1 $\beta$  + IFN- $\gamma$  failed to induce BiP mRNA, whereas thapsigargin induced a sixfold increase in BiP mRNA. The values for real-time RT-PCR of BiP/GAPDH were:  $27 \pm 3$  for control,  $30 \pm 7$  for

cytokines, and  $180 \pm 33$  for thapsigargin ( $P \leq 0.01$  for thapsigargin versus control,  $n = 3-7$ ).

ER stress activates PERK, leading to activation of the transcription factor ATF4 (5). ATF4 contributes to induce CHOP mRNA expression (34). In line with this, we observed a two- to fivefold induction of ATF4 mRNA expression in primary  $\beta$ -cells exposed for 6 or 24 h to IL-1 $\beta$  + IFN- $\gamma$  (Fig. 9A). A similar induction was observed after thapsigargin (Fig. 9A) or CPA (data not shown) treatment. Cytokine-induced ATF4 mRNA expression was prevented by the iNOS blocker LMA. IFN- $\gamma$  alone did not induce ATF4 expression, and LMA failed to prevent thapsigargin- or CPA-induced ATF-4 mRNA expression (data not shown). Cytokines induced a progressive increase in ATF4 mRNA expression in INS-1E cells, reaching significant levels at 8 h and peaking at 24 h, with a sevenfold increase as compared with control cells (Fig. 9B). CHOP expression followed a similar pattern. Both cytokine-induced ATF4 and CHOP mRNA expression in INS-1E cells were prevented by LMA (data not shown). These data are compatible with the hypothesis that ATF4 participates in the induction of CHOP expression.

## DISCUSSION

IL-1 $\beta$  + IFN- $\gamma$  induce the death of rodent and human  $\beta$ -cells mostly by apoptosis (10). Cytokine-triggered  $\beta$ -cell apoptosis depends on the activation of a complex network of transcription factors and effector genes (14-16). The transcription factor nuclear factor- $\kappa$ B and the mitogen-activated protein kinase/stress-activated protein kinase signaling seem to play a key role in this process (10,35,36), but it remains to be clarified how modifications in gene networks will transduce the signal(s) that activate the apoptotic program. The present study suggests that ER stress might be one component for induction of  $\beta$ -cell death. Cytokine-induced ER stress was prevented by the iNOS blocker LMA, indicating that it is mediated via iNOS activation and NO production. LMA, however, failed to prevent thapsigargin- or CPA-induced ER stress and  $\beta$ -cell apoptosis, confirming that the main effect of LMA is prevention of NO production. Of note, cytokine-induced iNOS expression is regulated by nuclear factor- $\kappa$ B (33).

Sensitivity of  $\beta$ -cells to NO-induced cell death varies with the species under study. Rat  $\beta$ -cells are more susceptible to cytokine-mediated NO formation than mouse or human  $\beta$ -cells (11-13,37). For both mouse (12) and rat islets (present data), cytokine-induced NO formation is the main determinant for necrosis, whereas cytokine-induced apoptosis has both an NO-dependent and an NO-independent component (10). This NO-dependent component is more pronounced in rat (current findings) than in mouse islet cells (12,13).

Chemical NO donors induce ER stress (as evaluated by CHOP expression) and apoptosis in the mouse insulinoma cell line MIN6 (8). To test whether cytokine-induced NO formation triggers an ER stress response in primary  $\beta$ -cells, we first analyzed IRE-1 $\alpha$ -mediated xbp-1 alternative splicing in these cells, an event specifically activated during ER stress (3). We found that IL-1 $\beta$ , alone or in combination with IFN- $\gamma$ , induces xbp-1 alternative splicing in  $\beta$ -cells, a phenomenon dependent on NO formation. This, together with the observed cytokine-triggered induc-

tion of CHOP mRNA expression, suggest that cytokines activate an ER stress response in pancreatic  $\beta$ -cells. Thapsigargin, a well-known inducer of ER stress in both  $\beta$ -cells and other cell types (3,17,18), also induced xbp-1 alternative splicing and CHOP expression in  $\beta$ -cells. It is intriguing that IL-1 $\beta$  alone induced xbp-1 alternative splicing, CHOP expression, and other components of the ER stress pathway (see below), but it failed to induce  $\beta$ -cell death in the absence of IFN- $\gamma$ . This suggests two possibilities that are not necessarily mutually exclusive: 1) ER stress is a necessary but not sufficient component for cytokine-induced  $\beta$ -cell death and 2) IFN- $\gamma$  aggravates and prolongs the ER stress induced by IL-1 $\beta$ . IFN- $\gamma$  by itself did not induce any of the different components of ER stress currently studied, but a previous microarray analysis has shown that IFN- $\gamma$  decreases the expression of several ER chaperones (38). This could decrease the  $\beta$ -cell capacity to cope with a prolonged ER stress and, together with the potentiating effect of IFN- $\gamma$  on IL-1 $\beta$ -induced iNOS expression (10), push  $\beta$ -cells to the "point of no return" in the pathway to apoptosis.

After establishing that cytokines, via NO production, trigger an ER stress response in  $\beta$ -cells, we next investigated the mechanisms involved in this effect. Because we have previously observed by microarray analysis that cytokines decrease mRNA expression of the ER  $\text{Ca}^{2+}$  pump SERCA2b (14–16), a finding confirmed in the current study at the mRNA and protein level, we focused on the possibility that cytokines, via NO formation, would lead to a depletion of ER  $\text{Ca}^{2+}$ . Of note, inhibition of SERCA2b by thapsigargin or CPA triggers ER stress and apoptosis in pancreatic  $\beta$ -cells (current data) (17,18), and  $\beta$ -cells are much more sensitive than fibroblasts to the proapoptotic effect of SERCA inhibition. We showed that cytokines severely deplete ER  $\text{Ca}^{2+}$  stores, using two complementary approaches, namely the determination of acute  $\text{Ca}^{2+}$  release from ER stores to the cytosol induced by thapsigargin (an indirect estimation of ER  $\text{Ca}^{2+}$  contents) (18,25) and a direct determination of ER  $\text{Ca}^{2+}$  concentration with furaptra (18,26). Blocking NO production with LMA prevented cytokine-induced SERCA2b inhibition, ER  $\text{Ca}^{2+}$  depletion, xbp-1 mRNA processing, CHOP expression, and  $\beta$ -cell death, suggesting that these processes are NO mediated. As suggested above, the mechanism by which cytokines/NO deplete ER  $\text{Ca}^{2+}$  probably involves downregulation of the ER  $\text{Ca}^{2+}$  pump SERCA2b, but we cannot exclude other mechanisms, such as alterations in the function of Ins(1,4,5)P $_3$  or ryanodine receptors. In line with this possibility, LMA only partially prevents cytokine-induced ER  $\text{Ca}^{2+}$  depletion.  $\beta$ -Cells express three isoforms of SERCA3 (a–c) and SERCA2b (39), but SERCA2b seems to be the crucial regulator of basal ER  $\text{Ca}^{2+}$  in  $\beta$ -cells (40). Of note, NO also inhibits ER  $\text{Ca}^{2+}$  uptake by SERCA pumps via a direct effect (41,42) and via generation of the radical peroxynitrite (43,44). Additional experiments are required to provide a "cause and effect" link between the observed decrease in SERCA2 expression and  $\beta$ -cell death.

Increased expression of CHOP mRNA is one of the mechanisms by which ER stress causes cell death (45,46), and we observed that cytokines induce CHOP mRNA and protein expression in  $\beta$ -cells via NO production. Induction

of CHOP protein, however, was observed in  $\beta$ -cells exposed to IL-1 $\beta$  alone, which is not sufficient to induce apoptosis. Islets from CHOP knockout mice are resistant to NO-induced cell death, but they are only partially protected against cytokine-induced apoptosis (8). These data suggest that CHOP induction is not the sole mediator of ER-triggered apoptosis in  $\beta$ -cells.

Another potential mechanism for ER stress-induced apoptosis is JNK activation (7), and cytokine-induced JNK activation has a proapoptotic role in insulin-producing cells (47). Although thapsigargin induced a progressive increase in JNK activation, with kinetics well correlated with activation of the other components of the ER stress pathway, cytokines induced an early and transitory JNK phosphorylation that preceded the subsequent ER stress. Thus, it seems that under the current experimental conditions, JNK is not a major executioner of ER stress-triggered  $\beta$ -cell death. It could, however, be an early and contributory signal for ER stress.

ER stress mobilizes PERK, leading to phosphorylation of eIF2 $\alpha$  and activation of the transcription factor ATF4 (5). Both ATF4 and ATF6 transactivate the CHOP promoter during ER stress response (5,34). Cytokines induced expression of ATF4, but they failed to activate an ATF6-regulated promoter. In contrast, thapsigargin induced both pathways in parallel. Against this background, it is conceivable that cytokine/NO induction of CHOP mRNA in  $\beta$ -cells is mostly mediated via ATF4. The observed differences between  $\beta$ -cell responses to thapsigargin and cytokines suggest that either cytokines induce an atypical ER stress response in these cells, characterized by a defective ATF6 activation, or that the signaling for the ER stress generated by cytokines is not sufficient to cross the putative threshold for activation of ATF6 expression. ATF6 regulates the expression of several key chaperones required for cellular recovery after ER stress (2). One of these chaperones is BiP, and, in line with the lack of cytokine-induced ATF6 activation, cytokines also failed to increase BiP mRNA expression. It is conceivable that the lack of ATF6 activation deprives the  $\beta$ -cells of one of the key mechanisms for cell survival during ER stress. If that is the case, this could contribute to the increased susceptibility of  $\beta$ -cells to cytokine- and NO-mediated ER stress.

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