

# Proinflammatory Cytokines Activate the Intrinsic Apoptotic Pathway in $\beta$ -Cells

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**OBJECTIVE**—Proinflammatory cytokines are cytotoxic to  $\beta$ -cells and have been implicated in the pathogenesis of type 1 diabetes and islet graft failure. The importance of the intrinsic mitochondrial apoptotic pathway in cytokine-induced  $\beta$ -cell death is unclear. Here, cytokine activation of the intrinsic apoptotic pathway and the role of the two proapoptotic Bcl-2 proteins, Bad and Bax, were examined in  $\beta$ -cells.

**RESEARCH DESIGN AND METHODS**—Human and rat islets and INS-1 cells were exposed to a combination of proinflammatory cytokines (interleukin-1 $\beta$ , interferon- $\gamma$ , and/or tumor necrosis factor- $\alpha$ ). Activation of Bad was determined by Ser136 dephosphorylation, mitochondrial stress by changes in mitochondrial metabolic activity and cytochrome c release, downstream apoptotic signaling by activation of caspase-9 and -3, and DNA fragmentation. The inhibitors FK506 and V5 were used to investigate the role of Bad and Bax activation, respectively.

**RESULTS**—We found that proinflammatory cytokines induced calcineurin-dependent dephosphorylation of Bad Ser136, mitochondrial stress, cytochrome c release, activation of caspase-9 and -3, and DNA fragmentation. Inhibition of Bad Ser136 dephosphorylation or Bax was found to inhibit cytokine-induced intrinsic proapoptotic signaling.

**CONCLUSIONS**—Our findings demonstrate that the intrinsic mitochondrial apoptotic pathway contributes significantly to cytokine-induced  $\beta$ -cell death and suggest a functional role of calcineurin-mediated Bad Ser136 dephosphorylation and Bax activity in cytokine-induced apoptosis. *Diabetes* 58: 1807–1815, 2009

**P**roinflammatory cytokines, particularly interleukin (IL)-1 $\beta$  in combination with interferon (IFN)- $\gamma$  and/or tumor necrosis factor (TNF)- $\alpha$ , have been implicated in the elimination of  $\beta$ -cells in type 1 diabetes (1). However, the exact molecular mechanisms by which proinflammatory cytokines induce

$\beta$ -cell death are not clear. While several signaling pathways have been suggested to play a role in cytokine-mediated cell death (1), it remains unclear how these pathways cooperate to induce apoptosis in  $\beta$ -cells. Furthermore, it is still debated whether cell death induced by proinflammatory cytokines occurs by necrosis, apoptosis, or both.

Apoptosis is an energy-dependent process in which dying cells activate a genetically encoded cell-death program. Essential in this process is the caspase family of cysteine proteases (2). Signals activating the “intrinsic apoptotic pathway” converge on the mitochondria, leading to mitochondrial membrane permeabilization and subsequent release of cytochrome c from the intermembranous space (3). Once in the cytosol, cytochrome c complexes with apoptosis-protease activating factor-1, which, in the presence of ATP, leads to activation of the initiator caspase, caspase-9. Through proteolytic cleavage, caspase-9 activates the effector caspase, caspase-3, leading to cleavage of numerous cellular targets and resulting in the systematic dismantling of the cell (4).

The release of cytochrome c from the mitochondria is regulated by the Bcl-2 family of proteins (5). The proapoptotic Bcl-2 proteins can be subdivided into “multidomain” and “BH3-only” proteins. Multidomain proapoptotic proteins, such as Bax and Bak, contain the conserved Bcl-2 homology (BH) domains 1–3. Upstream of the multidomain proteins are the BH3-only proapoptotic proteins, such as Bid and Bad, which contain only the amphipathic  $\alpha$ -helical BH3 domain. Antiapoptotic Bcl-2 family members contain all four BH domains (BH1–4) and are generally thought to prevent apoptosis by sequestering the proapoptotic Bcl-2 proteins, although the exact mechanisms remain elusive (6).

The proapoptotic multidomain proteins Bax and Bak are essential in mitochondrial dysfunction and cell death caused by a variety of stimuli (7). During apoptosis, Bax changes conformation and translocates from the cytosol to the mitochondria where it, along with Bak, mediates mitochondrial permeabilization and the release of cytotoxic proteins such as cytochrome c. Although the precise mechanism of mitochondrial permeabilization by proapoptotic Bcl-2 proteins is unclear, this step represents a critical checkpoint for survival, beyond which the cell is committed to death (3).

Under normal conditions, the BH3-only protein Bad is sequestered in the cytosol by binding to proteins of the 14-3-3 family of phospho-Ser/Thr-binding proteins, and this interaction is dependent upon phosphorylation of Bad at Ser112 and Ser136 (8). Following certain apoptotic stimuli, Bad is dephosphorylated, leading to its dissociation from 14-3-3. Dephosphorylated Bad can bind antiapoptotic proteins such as Bcl-X<sub>L</sub> or Bcl-2 and is thought to block their antiapoptotic function (8,9). The antiapop-

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See accompanying commentary, p. 1725.

otic/survival kinase Akt phosphorylates Bad at Ser136 (8,10), while the  $\text{Ca}^{2+}$ -dependent phosphatase calcineurin dephosphorylates this residue (and Ser112) in response to increased intracellular  $\text{Ca}^{2+}$  concentrations (11). In addition, the c-Jun  $\text{NH}_2$ -terminal kinase (JNK) can phosphorylate 14-3-3, resulting in 14-3-3/Bad dissociation, which is followed by Bad dephosphorylation and activation (12). Thus, the control of Bad phosphorylation provides a sensitive means of regulating cell survival (13).

There is accumulating evidence for a role of the mitochondrial pathway in proinflammatory cytokine-induced cell death in pancreatic  $\beta$ -cells. A combination of IL-1 $\beta$ , IFN- $\gamma$ , and TNF- $\alpha$  has been shown to induce mitochondrial membrane depolarization in rat RINm5F cells (14), and IL-1 $\beta$  induced cytochrome c release from the mitochondria and caspase-9 activity in rat islets (15). In addition, IFN- $\gamma$  and TNF- $\alpha$  induced Bad dephosphorylation at Ser112, release of cytochrome c, caspase activation, and cell death in mouse MIN6N8 cells (16). A role for the Bcl-2 proteins in cytokine-induced  $\beta$ -cell death has been suggested because the mRNA expression of several Bcl-2 members is regulated by IL-1 $\beta$  plus IFN- $\gamma$  exposure in INS-1E cells (17), IL-1 $\beta$  induces translocation of Bax out of the cytosol in rat islets (15), and overexpression of anti-apoptotic Bcl-2 members protects against the  $\beta$ -cell cytotoxic effects of proinflammatory cytokines (14,18–23).

It is not known how IL-1 $\beta$  potentiated by IFN- $\gamma$ /TNF- $\alpha$  signals to the mitochondria in  $\beta$ -cells. Following exposure of  $\beta$ -cells to IL-1 $\beta$  or IL-1 $\beta$  plus IFN- $\gamma$ , JNK activity and intracellular  $\text{Ca}^{2+}$  concentrations are increased (24–26), whereas Akt activity is decreased (27). These events may be crucial in IL-1 $\beta$ -induced proapoptotic signaling, since inhibition of JNK activity and  $\text{Ca}^{2+}$ -induced signaling prevent cytokine-induced  $\beta$ -cell death (26,28). Stimulation of Akt by survival factors (e.g., IGF) also protects  $\beta$ -cells from cytokine-induced apoptosis (27). However, the molecular mechanisms downstream of JNK, Akt, and  $\text{Ca}^{2+}$  in cytokine-induced  $\beta$ -cell death are not clear. The aim of this study was therefore to examine the contribution of the intrinsic mitochondrial pathway to cytokine-induced cell death and the role of Bax and Bad in this process.

## RESEARCH DESIGN AND METHODS

**Cell culture.** The clonal rat  $\beta$ -cell line INS-1 (a gift from Prof. Claes Wollheim, Geneva, Switzerland) was cultured at 37°C in a humidified atmosphere of 5%  $\text{CO}_2$  in complete RPMI-1640 medium (RPMI-1640 with glutamax [Invitrogen], 11 mmol/l glucose, 50  $\mu\text{mol/l}$   $\beta$ -mercaptoethanol, 100 units/ml penicillin, and 100  $\mu\text{g/ml}$  streptomycin) supplemented with 10% FBS. When confluent, cells were trypsinized and seeded for culture and experiments. In experiments with INS-1 cells and rat islets, IL-1 $\beta$  (160 pg/ml) and IFN- $\gamma$  (5 ng/ml) were used, while with human islets IL-1 $\beta$  (2 ng/ml), IFN- $\gamma$  (100 ng/ml), and TNF- $\alpha$  (100 ng/ml) were used. Recombinant mouse IL-1 $\beta$  was from BD Bioscience Pharmingen, and recombinant rat IFN- $\gamma$ , recombinant human IL-1 $\beta$ , recombinant human IFN- $\gamma$ , and recombinant human TNF- $\alpha$  were from R&D Systems. The combination and concentrations of cytokines were selected based on their ability to induce a four- to fivefold increase in apoptosis over background levels. The V5 Bax inhibitory peptide VPMLK (University of Calgary Peptide Synthesis, Calgary, Canada, and Calbiochem) was used at 100  $\mu\text{mol/l}$ . FK506 (Tacrolimus) from Sigma was used at 1  $\mu\text{mol/l}$ . In experiments examining the potentially protective effect of a compound, the compound was added 30–60 min prior to cytokines. For all compounds prepared in DMSO, the final concentration of DMSO in the culture media was kept below 0.1%. Appropriate vehicle controls were prepared for all treatments.

**Rat islet isolation.** Pancreata from 5- to 7-day-old outbred Wistar rats (Taconic) were collagenase digested, and islets were isolated by Percoll gradient purification (GE Healthcare). Five hundred or 1,000 islets were cultured for 5–7 days in complete RPMI-1640 medium with 10% FCS at 37°C in a humidified atmosphere of 5%  $\text{CO}_2$  before stimulation in complete RPMI-1640 medium with 0.5% human serum.

**Human islet isolation.** Pancreata were retrieved from heart-beating cadaveric donors at the time of multiorgan harvest for transplantation, and islets were isolated as described previously (28). The number of islet equivalents (IEQs) and islet purity were assessed using dithizone (Sigma). Glucose-stimulated insulin secretion was routinely assessed to ensure islet functionality. Isolated human islets (>85% purity) were cultured in CMRL-1066 medium (Life Technologies) containing 10% FBS (Wisent) at 37°C in a humidified atmosphere of 5%  $\text{CO}_2$ . Medium was changed every other day.

**Immunoblotting.** Immunoblotting was performed as described previously (24,28). Briefly, lysate protein concentrations were measured by the Bradford method according to the manufacturer's instructions (Bio-Rad). Equivalent amounts of protein from each condition were immunoblotted as described by the manufacturer (Invitrogen), and the protein of interest was detected by chemiluminescence. Anti-cytochrome c (BD Biosciences) was used at a dilution of 1:1,000; anti-cleaved caspase-3 (Cell Signaling Technology) at 1:250–1,000; anti-Bax (Santa Cruz Biotechnology) at 1:800; anti-cytochrome c oxidase subunit IV (COX4) (BD Biosciences) at 1:500; pBadS136 (Biosource) at 1:250; anti-phospho-JNK and total-JNK (Cell signaling) at 1:1,000; anti-actin (Abcam or Chemicon) at 1:10,000 or 1:1,000, respectively; anti-tubulin (Santa Cruz Biotechnology) at 1:1,000; and anti-cleaved caspase-9 (Cell Signaling Technology) at 1:500. Horseradish-peroxidase-conjugated anti-rabbit (1:2,000) and anti-mouse (1:1,000) were purchased from Cell Signaling Technology. Ponceau staining (Fluka biochemika) was used in Fig. 7E to show equal loading.

### Caspase-activity assays

**DEVDase assay.** To assess caspase-3-like protease activity, cleavage of the colorimetric substrate DEVD-pNA was measured as previously described (28). In short, the supernatant of islet sample lysates (2,000 IEQ) was used for analysis. Following determination of the protein content (Bio-Rad), the release of pNA was analyzed by measuring the absorbance at 405 nm using a Benchmark Microplate Reader (Bio-Rad).

**Caspase-9 assay.** To assess caspase-9 activity, luciferase activity dependent on caspase-9-mediated generation of luciferase substrate was measured. Briefly, 25 rat islets or  $1 \times 10^6$  INS cells were exposed to cytokines for 24 h. Caspase-Glo reagent (Caspase-Glo; Promega) was added directly to islet cultures or to INS cells following removal of the medium, and light emission was detected after 30 min using a VictorX Light (PerkinElmer).

### 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

Aliquots containing 500 IEQ in 500  $\mu\text{l}$  of medium were placed in sterile Eppendorf tubes, and 50  $\mu\text{l}$  of stock 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (5 mg/ml) (Sigma) was added to each sample. The samples were incubated at 37°C for 2 h, washed twice with cold PBS, and lysed with 200  $\mu\text{l}$  of DMSO (Sigma). Aliquots of 100  $\mu\text{l}$  from each sample were transferred to a 96-well plate, and the absorbance was measured at 595 nm using a Benchmark Microplate Reader (Bio-Rad). Four independent samples were analyzed per experiment, and each experiment was repeated at least three times.

### Insulin assay

For measurement of glucose-stimulated insulin release, cultured human islets (100 IEQ per group in duplicate) were washed with CMRL-1066 and incubated in Hank's buffered saline containing 2.2 mmol/l glucose for two consecutive periods of 60 min at 37°C. Next, islets were incubated for 30 min with 22 mmol/l glucose and then another 30 min with 22 mmol/l glucose and 50  $\mu\text{mol/l}$  3-isobutyl-1-methylxanthine (IBMX), an inhibitor of cyclic AMP phosphodiesterase. Finally, islets were washed with Hank's buffered saline and incubated for 1 h in 2.2 mmol/l glucose. The supernatants were kept following each incubation and analyzed for insulin content using a commercially available insulin enzyme-linked immunosorbent assay kit (Crystal Chem). Insulin release was normalized to the protein content of the pellet determined using a Bio-Rad Protein Assay Dye Reagent. For measurement of accumulated insulin release, 150 rat islets were cultured in 1 ml of medium. Following 24 h of cytokine exposure, accumulated insulin in the incubation medium was measured as previously described (29).

### Subcellular fractionation

Cytosolic and mitochondria-enriched fractions were prepared using an ApoAlert Cell Fractionation Kit according to the manufacturer's directions (BD Biosciences). Briefly, islet samples (8,000 IEQ) were resuspended in fractionation buffer (BD Biosciences) and incubated on ice for 10 min. Cells were then homogenized on ice using 100 passes with a dounce tissue grinder. The resulting homogenate was centrifuged at 3,000 rpm for 10 min, and the supernatant was kept. The supernatant was centrifuged at 12,000 rpm for 25 min, and the resulting supernatant was the cytosolic fraction. The mitochondria-enriched pellet was resuspended in 10  $\mu\text{l}$  fractionation buffer. The protein content was determined using a Bio-Rad Protein Assay Dye Reagent.

### Cell death detection assay

As a marker for apoptosis, histone/DNA complexes released from the nucleus to the cytosol were measured according to the manufacturer's instructions

(Cell Death Detection ELISA<sup>PLUS</sup>; Roche). In short, cell lysates were incubated with anti-DNA-peroxidase and anti-histone-biotin and transferred to streptavidin-coated wells. Absorbance was measured after addition of the peroxidase substrate ABTS (2',2'-azino-bis-3-ethylbenzthiazoline-6-sulfonate).

#### Statistical analysis

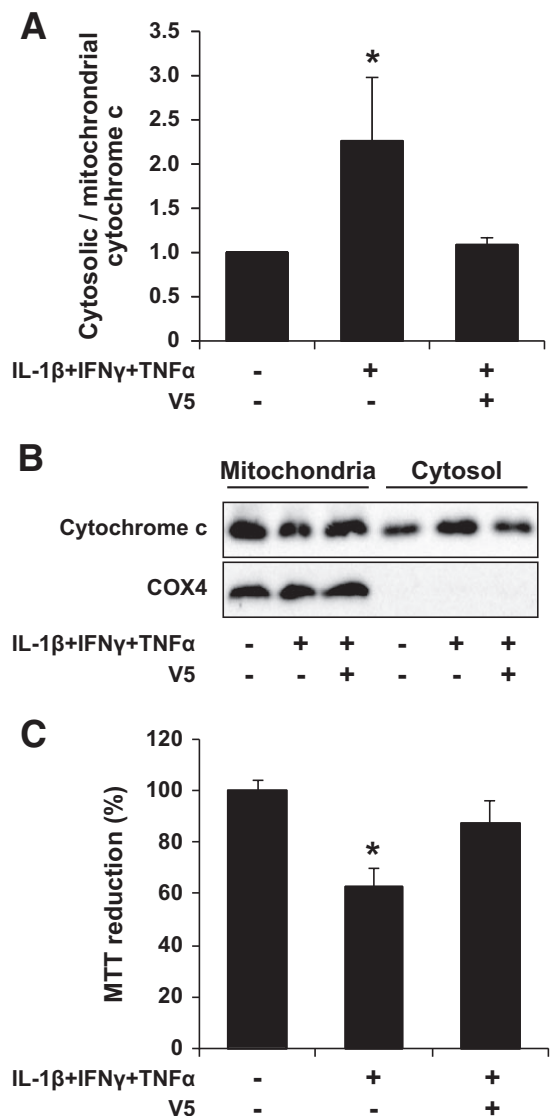
Data are presented as means  $\pm$  SE. Statistical significance was determined by Student's paired *t* test (on the planned comparisons reported) or by two-way ANOVA with Dunnett's post hoc test (time course experiments) (Figs. 3A and 5A), with *P* < 0.05 as the level of significance.

## RESULTS

**Cytokines induce Bax-dependent mitochondrial stress.** To investigate if cytokines induce mitochondrial stress in human islets, we investigated the effects of cytokine exposure on mitochondrial cytochrome c release and mitochondrial metabolic activity. We used cell fractionation followed by immunoblotting to observe a movement of cytochrome c from the mitochondrial fraction into the cytosolic fraction after cytokine exposure (Fig. 1A and B). Immunoblotting for COX4 was used to confirm that the cytosolic fractions were not contaminated with mitochondrial proteins. Cytokine exposure also led to a significant decrease in mitochondria metabolic activity examined with the MTT assay (Fig. 1C).

To examine if cytokine-mediated mitochondrial dysfunction is mediated by Bax, we used the Bax inhibitory peptide V5. The V5 peptide is derived from the Bax-binding domain of human Ku70 (30). Ku70 binds Bax in the cytosol and prevents the proapoptotic functions of Bax by inhibiting Bax translocation to the mitochondria (31,32). In analogy, V5 binds Bax, inhibits Bax activation (proapoptotic conformational change), and suppresses apoptosis (30,33). To confirm that V5 can efficiently enter cells within an islet, isolated human islets were treated with 5-FAM (6-carboxy-fluorescein)-labeled V5. When treated islets were washed and dispersed into single cells, all cells were found to contain the labeled peptide localized mainly to the cytosol (confocal microscopy) (data not shown). Immunoblot analysis of cytosolic and mitochondria-enriched fractions showed that V5 decreased cytokine-induced cytochrome c release from the mitochondria into the cytosol (Fig. 1A). V5 was also able to protect human islets against cytokine-induced inhibition of mitochondrial function (Fig. 1B), whereas a negative control peptide had no effect (Calbiochem) (data not shown). Taken together, these findings demonstrate that inflammatory cytokines induce Bax-mediated mitochondrial dysfunction and cytochrome c release.

**V5 does not affect glucose-stimulated insulin release but reduces cytokine-mediated inhibition of accumulated insulin release.** Perturbations in mitochondrial function and integrity can lead to defective insulin secretion (34). Overexpression of Bcl-X<sub>L</sub> has previously been shown to impair insulin secretion and was associated with altered mitochondrial polarization and Ca<sup>2+</sup> signaling in response to glucose (35). We therefore examined whether V5 affected glucose-stimulated insulin release in isolated human islets. Freshly isolated islets were cultured for 48 h in the presence of 100  $\mu$ M V5, and glucose-stimulated insulin release was assessed. V5 had no effect on basal insulin release at low glucose levels or on insulin release in response to high glucose (Fig. 2A). Addition of IBMX, an inhibitor of cyclic AMP phosphodiesterase, causes elevated intracellular cyclic AMP levels that lead to increased exocytosis of insulin (36). IBMX-induced insulin release was unaffected by V5 treatment (Fig. 2A). Furthermore, V5 did not affect regulation of insulin secretion following a

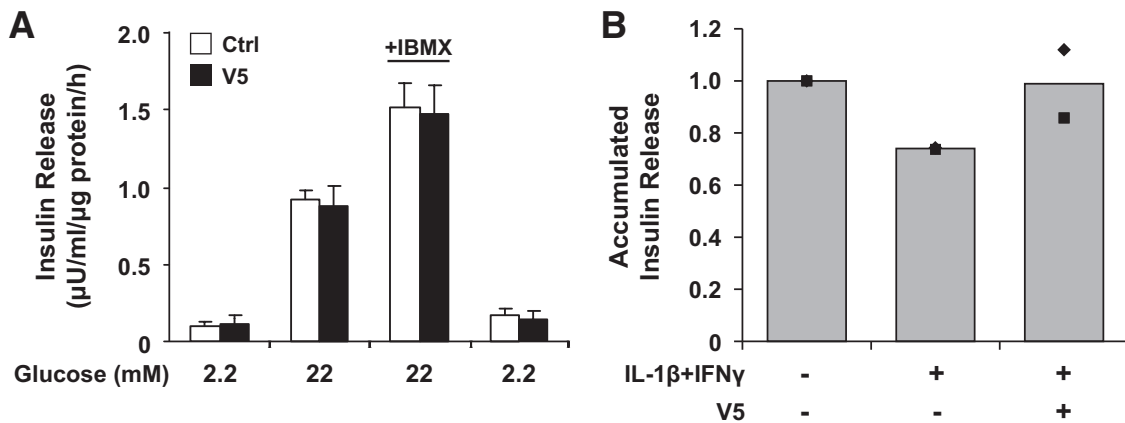


**FIG. 1.** Cell-permeable V5 inhibits cytokine-induced cytochrome c release and improves mitochondrial metabolic activity in human islets. Cells were pre-exposed to V5 (100  $\mu$ M) for 1 h before exposure to cytokines for 24 h. **A** and **B**: Immunoblotting was performed on fractionated cell lysates from human islets to assess the cellular localization of cytochrome c. COX4, a mitochondrial protein, was used as a control for the fractionation. Quantifications are shown as the ratio of cytosolic to mitochondrial cytochrome c (**A**). Representative gels from three independent experiments are shown (**B**). **C**: Changes in the mitochondrial activity in human islets were measured with the MTT assay as described in RESEARCH DESIGN AND METHODS. Bars represent means  $\pm$  SE for five independent experiments. \**P* < 0.05, \*\**P* < 0.01 vs. treatment without cytokines.

return to low glucose levels (Fig. 2A). Therefore, V5 did not adversely affect glucose-stimulated insulin secretion from isolated human islets.

It has been demonstrated previously that cytokines inhibit insulin release (1). Having established that V5 did not affect glucose-stimulated insulin release, we next asked whether V5 affected cytokine-mediated inhibition of insulin release from rat islets. Thus, the effect of cytokines on accumulated insulin in the culture medium released from rat islets during 24 h incubation in the presence or absence of V5 was measured. As seen in Fig. 2B, the inhibitory effect of cytokines on accumulated insulin release was reduced by V5.





**FIG. 2.** V5 does not affect glucose-stimulated insulin release but reduces cytokine-mediated inhibition of accumulated insulin release. **A:** Isolated human islets were cultured with or without the addition of V5 (100 μmol/l) to the culture media. Glucose-stimulated insulin release was examined after incubation with 2.2 mmol/l glucose for two consecutive periods of 60 min followed by incubation with 22 mmol/l glucose for 30 min, then another 30 min with 22 mmol/l glucose and 50 μmol/l isobutylmethylxanthine (IBMX), and finally after 1 h in 2.2 mmol/l glucose (see RESEARCH DESIGN AND METHODS for details). Bars represent means ± SE for three independent experiments. **B:** Rat islets were exposed to cytokines with or without V5 (added 1 h prior to cytokine exposure), and accumulated insulin was measured after 24 h as described in RESEARCH DESIGN AND METHODS. Bars represent mean values for two independent experiments, and results from the individual experiments are indicated.

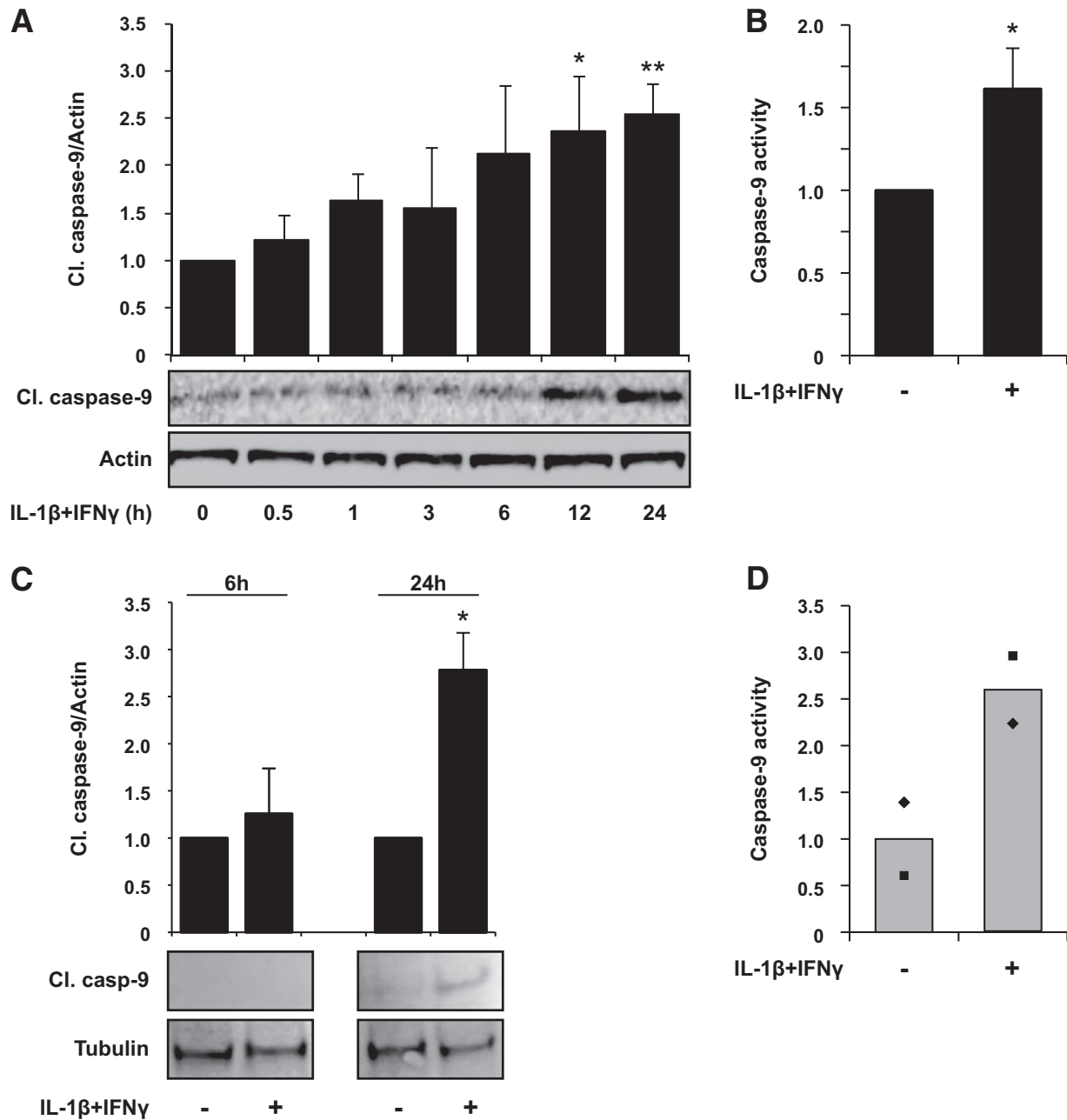
**Combination of IL-1β and IFN-γ induces cleavage and activation of caspase-9.** To verify the cytokine-induced activation of caspase-9 in INS-1 cells and rat islets, we used an antibody specific for cleaved caspase-9 and a caspase-9 activity assay. The combination of IL-1β and IFN-γ caused a time-dependent cleavage of caspase-9 in INS-1 cells, which was significant after 12 h of cytokine exposure (Fig. 3A). In rat islets, caspase-9 was cleaved with slower kinetics than in INS-1 cells (Fig. 3C). In both INS-1 cells (Fig. 3B) and rat islets (Fig. 3D), cleavage of caspase-9 was associated with increased caspase-9 activity. In contrast, we observed that caspase-8 was activated not by the combination of IL-1β and IFN-γ but only when TNF-α was added to this combination (data not shown). **Cytokine-induced β-cell toxicity is inhibited by V5.** We observed caspase-3 cleavage after 24 h of cytokine exposure in INS-1 cells (Fig. 4A) and in human islets (Fig. 4B). To assess if caspase-3 activation depended upon Bax activity, we examined the effect of the V5 inhibitor on caspase-3 cleavage and DEVDase activity, indicative of caspase-3-like protease activity. In both human islets and INS-1 cells, cytokine-induced processing of caspase-3 to its cleaved (active) form was inhibited by pre-exposure to V5 for 1 h (Fig. 4A and B). Cytokine treatment for 24 h resulted in a nearly fourfold increase in DEVDase activity, and this effect was inhibited by V5 pretreatment (Fig. 4C). Furthermore, pre-exposure to V5 decreased cytokine-induced oligonucleosomal fragmentation after 24 h—a marker of apoptosis (Fig. 4D). Taken together, these results suggest that Bax-mediated mitochondrial permeabilization is important in cytokine-induced islet cell death.

**Cytokines induce dephosphorylation of Bad Ser136.** To examine the role of Bad in cytokine-induced Bax activation and apoptosis signaling, we investigated the effects of cytokine exposure on Bad Ser136 dephosphorylation. In INS-1 cells, IL-1β plus IFN-γ induced a time-dependent dephosphorylation of Bad Ser136 (Fig. 5A) without affecting Bad protein expression (data not shown). In rat islets, exposure to IL-1β plus IFN-γ induced Bad Ser136 dephosphorylation (Fig. 5B) with slower kinetics than in INS-1 cells. We were unable to detect phospho-Bad Ser136 in human islets (data not shown). In

line with the fact that Bad acts upstream of Bax activation (3), we found that cytokine-induced Bad Ser136 dephosphorylation was unaffected by V5 (Fig. 5C).

**Calcineurin is involved in cytokine-induced Bad dephosphorylation.** The Ca<sup>2+</sup>-activated Ser/Thr phosphatase calcineurin dephosphorylates Bad, leading to induction of apoptosis (11). To investigate if IL-1β + IFN-γ-induced dephosphorylation of Bad Ser136 was mediated by calcineurin, we pre-exposed β-cells to the calcineurin inhibitor FK506 1 h before cytokine stimulation for 24 h. As seen in Fig. 6, FK506 inhibited IL-1β + IFN-γ-induced dephosphorylation of Bad Ser136 in both INS-1 cells (Fig. 6A) and rat islets (Fig. 6B), suggesting that calcineurin is important in IL-1β + IFN-γ-induced dephosphorylation of Bad Ser136. Since increased intracellular Ca<sup>2+</sup> concentrations induce calcineurin activity (37), we examined the effect of Ca<sup>2+</sup> influx induced by KCl. We found that a 24-h exposure to KCl (50 mmol/l) induced dephosphorylation of Bad Ser136 equal to that induced by IL-1β + IFN-γ in INS-1 cells but did not affect IL-1β + IFN-γ-induced Bad Ser136 dephosphorylation (data not shown). These data support a role of calcineurin in Bad Ser136 dephosphorylation.

**Inhibition of calcineurin decreases cytokine-induced β-cell death.** To investigate if calcineurin-mediated Bad dephosphorylation causes cytokine-induced cell death, we examined whether pre-exposure to FK506 inhibited cytokine-induced β-cell death. In rat islets, FK506 inhibited cytokine-induced cleavage of caspase-3 (Fig. 7A). Accordingly, FK506 decreased cytokine-induced DEVDase activity in human islets (Fig. 7B). FK506 also improved mitochondrial function and decreased apoptosis caused by cytokine exposure in human islets (Fig. 7C and D). To demonstrate that the protective effect of FK506 was not due to inhibition of JNK, as has been observed elsewhere (38), we examined the effect of FK506 on JNK phosphorylation, which is necessary for IL-1β-induced apoptosis (1). As shown in Fig. 7E, FK506 did not affect cytokine-induced JNK phosphorylation or JNK expression. Taken together, these findings demonstrate that proinflammatory cytokines induce calcineurin-mediated dephosphorylation of Bad Ser136, leading to cell death.



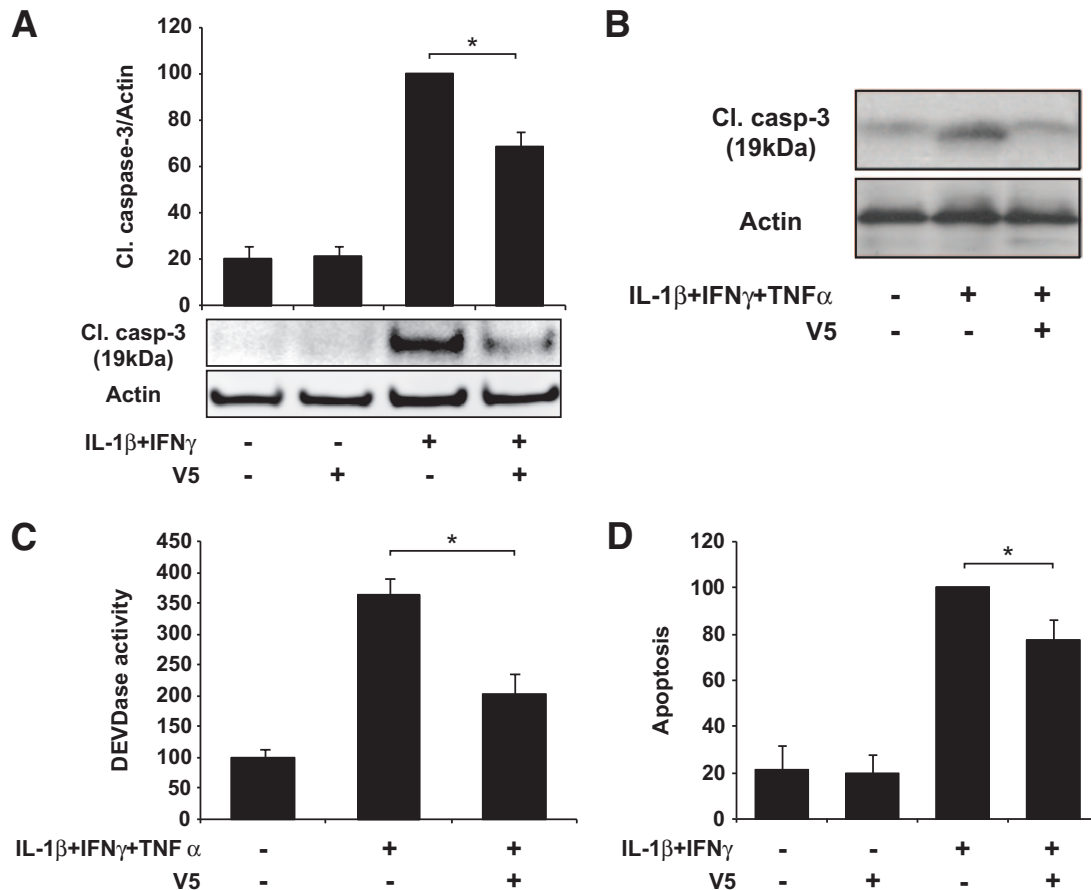
**FIG. 3.** Cytokines induce caspase-9 activation in INS-1 cells and rat islets. Cleavage of caspase-9 after cytokine exposure was assessed by immunoblotting in INS-1 cells (A) and rat islets (C). Representative gels of five independent experiments (A) or three independent experiments (C) are shown; results were adjusted for actin and tubulin levels. Caspase-9 activity in INS-1 cells ( $n = 5$ ) (B) and rat islets ( $n = 2$ ) (D) in the presence or absence of cytokines was measured as described in RESEARCH DESIGN AND METHODS. Bars represent means  $\pm$  SE (A, B, and C) or means with indication of the data from the individual experiments (D). \* $P < 0.05$ , \*\* $P < 0.01$  vs. the control.

## DISCUSSION

In this study, we demonstrate that cytokines induce 1) dephosphorylation of Bad on Ser136 in INS-1 and rat islet cells, 2) Bax-dependent release of cytochrome c and inhibition of mitochondrial metabolic activity in human islets, 3) cleavage/activation of caspase-9 in INS-1 cells and rat islets and cleavage/activation of caspase-3 in INS-1 cells and rat and human islets, and finally 4)  $\beta$ -cell apoptosis in INS-1 cells and rat and human islets. Taken together, our findings strongly suggest that cytokines induce  $\beta$ -cell apoptosis through the canonical intrinsic mitochondrial pathway.

It has been suggested that cytokine-induced  $\beta$ -cell death

is independent of Bax or Bak since siRNA-mediated knockdown of Bax or Bak failed to affect cytokine-induced cell death (39). In that study, the combination of IL-1 $\beta$  and IFN- $\gamma$  was found to induce cell death in INS-1-derived cell lines and rat islets via nonapoptotic killing, which is in contrast to our present observations. However, the INS-1-derived cells used previously (39) were stably transfected with the human proinsulin gene and were selected for G418 resistance (40), which may well affect the sensitivity to cytokines, as observed by Chen et al. (41). Another discrepancy between the studies is the markedly higher concentrations of cytokines used by Collier et al. (39) than in our study, which may preferen-



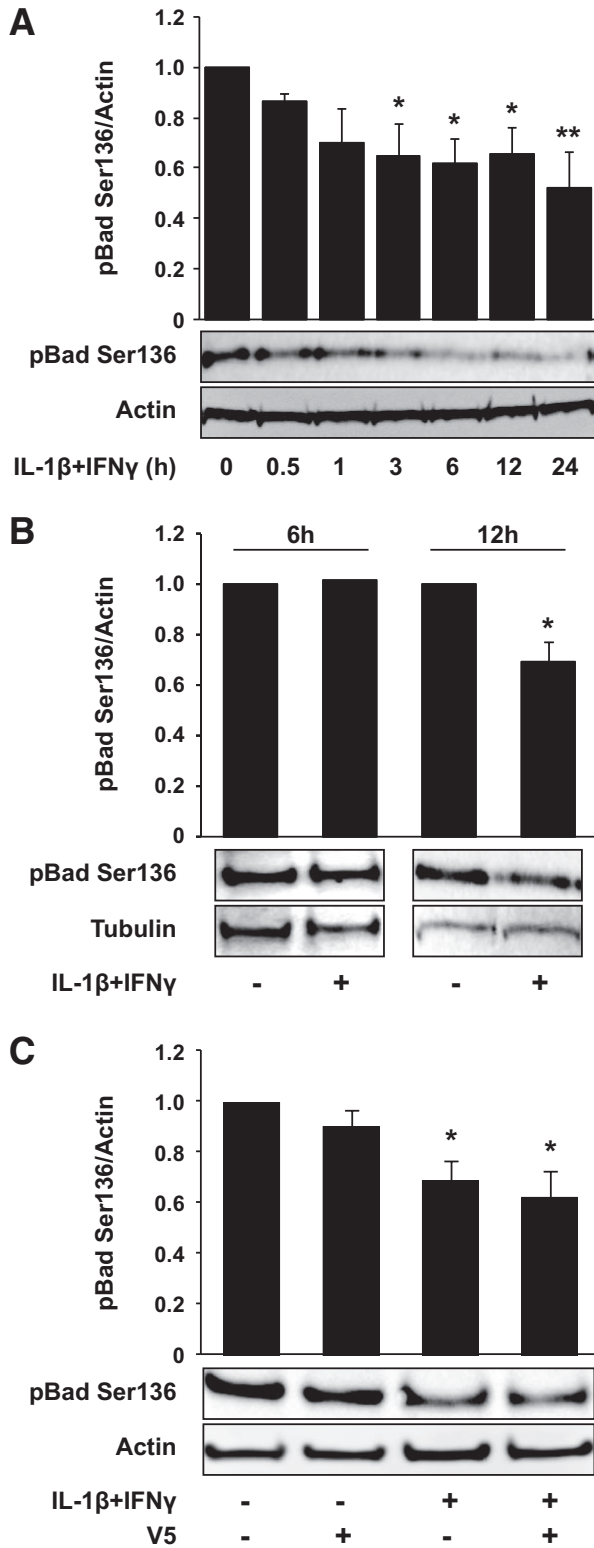
**FIG. 4.** V5 inhibits cytokine-induced cleavage of caspase-3 in INS-1 cells and human islets, DEVDase activity in human islets, and apoptosis in rat islets. Cells were pre-exposed to V5 (100  $\mu\text{mol/l}$ ) for 1 h before exposure to cytokines for 24 h. **A:** Cleaved caspase-3 in INS-1 cells was assessed with immunoblotting. Bars represent mean  $\pm$  SE of six independent experiments; representative gels are shown (actin was used as a loading control). **B:** Cleaved caspase-3 in human islet cells. Representative gels of three independent experiments are shown (actin was used as a loading control). **C:** DEVDase activity in human islets was measured as described in RESEARCH DESIGN AND METHODS. Bars represent mean values of three independent experiments. **D:** Rat islet apoptosis was measured as cytosolic appearance of nucleosomes as described in RESEARCH DESIGN AND METHODS. Bars represent mean  $\pm$  SE of eight independent experiments performed in rat islets. \* $P < 0.05$ .

tially induce nonapoptotic  $\beta$ -cell killing (e.g., via reactive oxygen species/nitric oxide [NO]), while  $\beta$ -cell apoptosis may primarily be induced at lower concentrations. Thus, Bax could be required for cytokine-induced  $\beta$ -cell apoptosis but not for cytokine-induced nonapoptotic  $\beta$ -cell killing. Further studies are needed to clarify the relative importance of apoptosis versus necrosis, which may depend on the model system used and the particular combination and concentration of proinflammatory cytokines.

A certain functional redundancy exists between Bax and Bak (7). Thus, while we show that inhibition of Bax alone decreased cytokine-induced  $\beta$ -cell death, the combined targeting of Bax and Bak may afford greater protection. In addition to the intrinsic pathway investigated in this study, apoptosis may also be induced by activation of the extrinsic (death receptor induced) pathway through caspase-8-mediated caspase-3 cleavage. In  $\beta$ -cells, TNF- $\alpha$ , but not IL-1 $\beta$ , induces activation of caspase-8 (15,42), indicating that TNF- $\alpha$  is able to activate the extrinsic pathway. In agreement with this, we observed that caspase-8 is activated by TNF- $\alpha$  + IL-1 $\beta$  + IFN- $\gamma$  but not by IL-1 $\beta$  + IFN- $\gamma$  only (data not shown). In some cell types, the extrinsic pathway must activate the intrinsic pathway to induce cell death through caspase-8-mediated cleavage of the BH3-only Bcl-2 protein Bid. Bid is required for the potentiating effects of TNF- $\alpha$  on IL-1 $\beta$  + IFN- $\gamma$ -induced DNA fragmen-

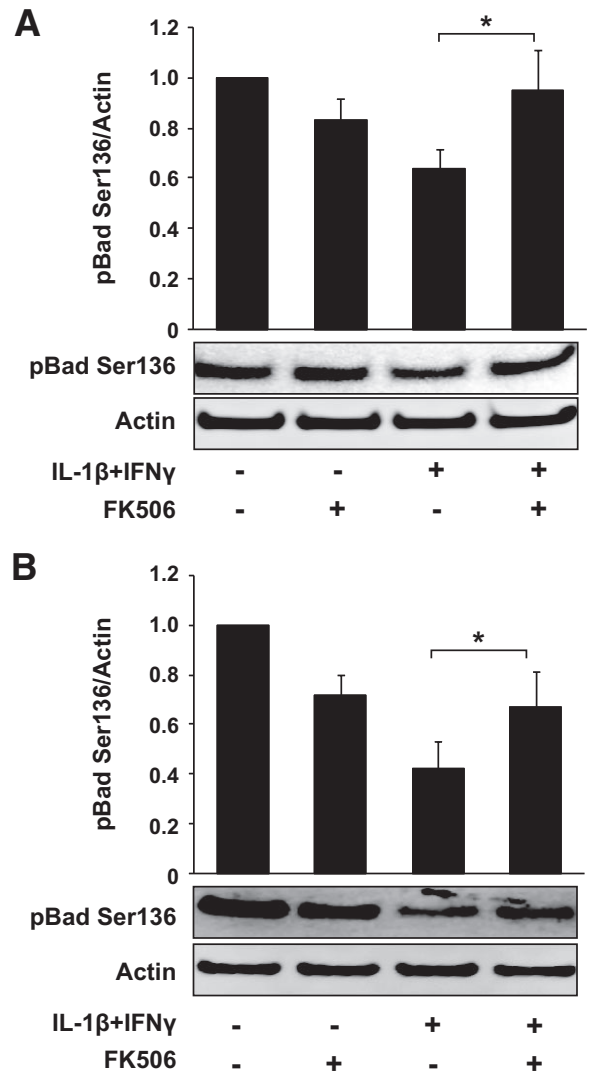
tation in primary mouse islets (43), indicating that the intrinsic pathway is important for TNF- $\alpha$ -mediated  $\beta$ -cell death. Overall, the relative dependence of cytokine-induced cell death on the extrinsic and intrinsic pathways remains unclear, mostly due to an incomplete understanding of how these pathways interact.

Because of its regulation by both prosurvival and apoptotic signals, Bad may act as a survival switch between  $\beta$ -cell death and survival. Thus, in other cell systems, antiapoptotic Akt signaling induces Bad Ser136 phosphorylation and inactivation (13), whereas proapoptotic JNK signaling and increased intracellular  $\text{Ca}^{2+}$  concentration induce Bad Ser136 dephosphorylation and activation (11,12). This notion may be of special relevance in cytokine-induced  $\beta$ -cell death because cytokines decrease Akt activity (27), induce JNK activity (28), and increase intracellular  $\text{Ca}^{2+}$  concentration (26). In this study, the FK506 inhibitor is used to abrogate cytokine-induced Bad dephosphorylation mediated by calcineurin. In other cell systems, FK506 inhibits nuclear factor  $\kappa\text{B}$ -induced expression of inducible NO synthase (44). In rodent islets, L-arginine analogues that block inducible NO synthase activity inhibit cytokine-induced  $\beta$ -cell death (1). However, NO plays only a minor role in cytokine-induced apoptosis in human islets (1), and the molecular mechanism underlying the protective effect of FK506 against



**FIG. 5.** IL-1 $\beta$  plus IFN- $\gamma$  induces Bax-independent dephosphorylation of Bad Ser136 in INS-1 cells and rat islets. Bad Ser136 dephosphorylation after cytokine exposure was assessed by immunoblotting in INS-1 cells (A and C) or rat islets (B). C: Cells were pre-exposed to V5 (100  $\mu$ mol/l) for 1 h before exposure to cytokines for 24 h. Data are presented as mean  $\pm$  SE of four experiments (A), seven experiments (24 h) (B) or two experiments (6 h) (B), and seven experiments (C). Representative gels are shown, and actin and tubulin were used as loading controls. \* $P$  < 0.05, \*\* $P$  < 0.01, \*\*\* $P$  < 0.001 vs. controls.

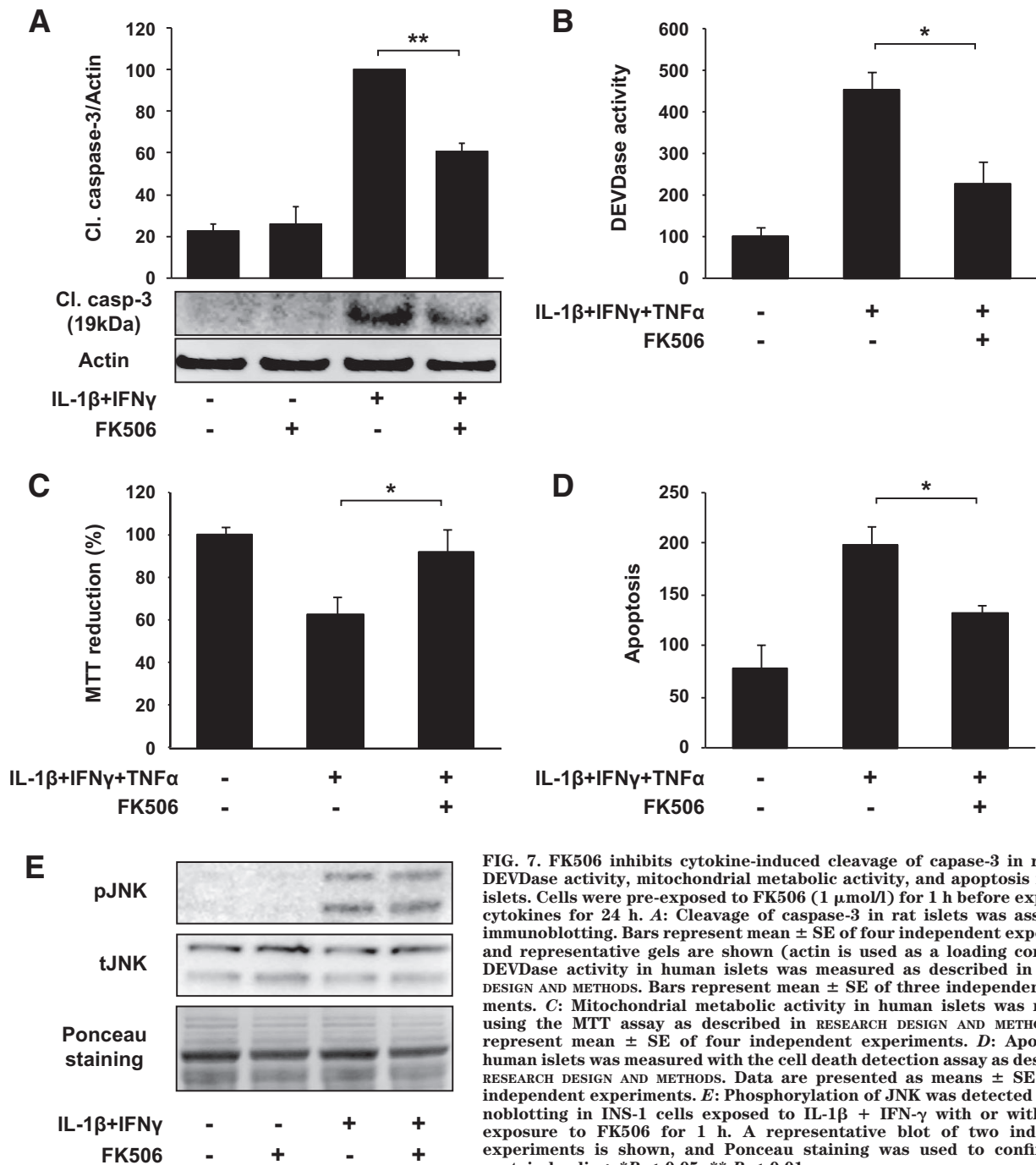
cytokine-induced apoptosis in human islets is therefore most likely independent of inhibition of NO synthesis.



**FIG. 6.** FK506 inhibits cytokine-induced dephosphorylation of Bad Ser136 in INS cells and rat islets. Cells were pre-exposed to FK506 (1  $\mu$ mol/l) for 1 h before exposure to cytokines for 24 h. Phosphorylation of Bad Ser136 in INS-1 cells (A) and rat islets (B) was assessed by immunoblotting. Bars represent mean  $\pm$  SE of four independent experiments, and representative gels are shown. (Actin was used as loading controls.) \* $P$  < 0.05.

Exposure of  $\beta$ -cells to a combination of IL-1 $\beta$  and IFN- $\gamma$  increases cytosolic Ca<sup>2+</sup> levels via low-voltage-activated Ca<sup>2+</sup> channels (26). Antagonists of Ca<sup>2+</sup> channels prevent the rise in cytosolic Ca<sup>2+</sup> and reduce cell death in cytokine-treated  $\beta$ -cells (16,26), suggesting a key role of Ca<sup>2+</sup> in cytokine-induced cell death. Indeed, previous findings suggested that IFN- $\gamma$ - and TNF- $\alpha$ -induced cell death may be partially mediated by Ca<sup>2+</sup>-activated proteins such as calpain or calcineurin (16). Our findings suggest that calcineurin-mediated dephosphorylation of Bad Ser136 may provide a link between the rise in cytosolic Ca<sup>2+</sup> and the core death machinery in  $\beta$ -cells upon cytokine exposure.

Calcineurin-based immunosuppressants are known to impair insulin production by blocking calcineurin-mediated activation of the transcription factor nuclear factor of activated T-cells (NFAT), and long-term exposure is associated with decreased  $\beta$ -cell survival (45–49). We have previously suggested that the long-term negative effect of calcineurin-based inhibitors on  $\beta$ -cell survival



**FIG. 7.** FK506 inhibits cytokine-induced cleavage of caspase-3 in rat islets, DEVDase activity, mitochondrial metabolic activity, and apoptosis in human islets. Cells were pre-exposed to FK506 (1  $\mu$ mol/l) for 1 h before exposure to cytokines for 24 h. **A:** Cleavage of caspase-3 in rat islets was assessed by immunoblotting. Bars represent mean  $\pm$  SE of four independent experiments, and representative gels are shown (actin is used as a loading control). **B:** DEVDase activity in human islets was measured as described in RESEARCH DESIGN AND METHODS. Bars represent mean  $\pm$  SE of three independent experiments. **C:** Mitochondrial metabolic activity in human islets was measured using the MTT assay as described in RESEARCH DESIGN AND METHODS. Bars represent mean  $\pm$  SE of four independent experiments. **D:** Apoptosis in human islets was measured with the cell death detection assay as described in RESEARCH DESIGN AND METHODS. Data are presented as means  $\pm$  SE of three independent experiments. **E:** Phosphorylation of JNK was detected by immunoblotting in INS-1 cells exposed to IL-1 $\beta$  + IFN- $\gamma$  with or without pre-exposure to FK506 for 1 h. A representative blot of two independent experiments is shown, and Ponceau staining was used to confirm equal protein loading. \* $P$  < 0.05; \*\* $P$  < 0.01.

may be due to the inhibition of autocrine insulin-mediated survival (50). In the current study, we exposed  $\beta$ -cells to FK506 for a relatively short time period (25 h), and the rationale for using FK506 in this study was solely to examine its role on calcineurin-mediated Bad dephosphorylation. Thus, it is entirely possible that the short-term protective effects of FK506 against cytokine-mediated apoptosis are overshadowed by long-term toxicity.

In summary, our findings demonstrate that proinflammatory cytokines induce  $\beta$ -cell apoptosis through the canonical mitochondrial pathway and that this effect is dependent on calcineurin-mediated Bad dephosphorylation and Bax activity. These results enhance our understanding of proapoptotic signaling induced by proinflammatory cytokines in  $\beta$ -cells and may provide the

basis for the development of future strategies for intervention in type 1 diabetes and islet graft failure.

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## REFERENCES

- Eizirik DL, Mandrup-Poulsen T. A choice of death: the signal-transduction of immune-mediated beta-cell apoptosis. *Diabetologia* 2001;44:2115–2133
- Hengartner MO. The biochemistry of apoptosis. *Nature (London)* 2000;407:770–776
- Daniel NN, Korsmeyer SJ. Cell death: critical control points. *Cell* 2004;116:205–219
- Tibbetts MD, Zheng L, Lenardo MJ. The death effector domain protein family: regulators of cellular homeostasis. *Nat Immunol* 2003;4:404–409
- Scorrano L, Korsmeyer SJ. Mechanisms of cytochrome c release by proapoptotic BCL-2 family members. *Biochem Biophys Res Commun* 2003;304:437–444
- Adams JM. Ways of dying: multiple pathways to apoptosis. *Genes Dev* 2003;17:2481–2495
- Wei MC, Zong WX, Cheng EHY, et al. Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. *Science* 2001;292:727–730
- Zha J, Harada H, Yang E, et al. Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not Bcl-X<sub>L</sub>. *Cell* 1996;87:619–628
- Yang E, Zha J, et al. Bad, a heterodimeric partner for Bcl-x(L), and Bcl-2, displaces Bax and promotes cell death. *Cell* 1995;80:285–291
- Peso LD, Gonzalez-Garcia M, Page C, et al. Interleukin-3-induced phosphorylation of BAD through the protein kinase Akt. *Science* 1997;278:687–689
- Wang HG, Pathan N, Ethell IM, et al. Ca<sup>2+</sup>-induced apoptosis through calcineurin dephosphorylation of BAD. *Science* 1999;284:339–343
- Sunayama J, Tsuruta F, Masuyama N, et al. JNK antagonizes Akt-mediated survival signals by phosphorylating 14–3-3. *J Cell Biol* 2005;170:295–304
- Datta SR, Brunet A, Greenberg ME. Cellular survival: a play in three Akts. *Genes Dev* 1999;13:2905–2927
- Barbu A, Welsh N, Saldeen J. Cytokine-induced apoptosis and necrosis are preceded by disruption of the mitochondrial membrane potential ( $\Delta\psi(m)$ ) in pancreatic R1Nm5F cells: prevention by Bcl-2. *Mol Cell Endocrinol* 2002;190:75–82
- Papaccio G, Graziano A, Valiante S, et al. Interleukin (IL)-1 $\beta$  toxicity to islet  $\beta$  cells: efazoxan exerts a complete protection. *J Cell Physiol* 2005;203:94–102
- Chang I, Cho N, Kim S, et al. Role of calcium in pancreatic islet cell death by IFN- $\gamma$ /TNF- $\alpha$ . *J Immunol* 2004;172:7008–7014
- Kutlu B, Cardozo AK, Darville MI, et al. Discovery of gene networks regulating cytokine-induced dysfunction and apoptosis in insulin-producing INS-1 cells. *Diabetes* 2003;52:2701–2719
- Saldeen J. Cytokines induce both necrosis and apoptosis via a common Bcl-2-inhibitable pathway in rat insulin-producing cells. *Endocrinology* 2000;141:2003–2010
- Klein D, Ribeiro MM, Mendoza V, et al. Delivery of Bcl-XL or its BH4 domain by protein transduction inhibits apoptosis in human islets. *Biochem Biophys Res Commun* 2004;323:473–478
- Liu Y, Rabinovitch A, Suarez-Pinzon W, et al. Expression of the bcl-2 gene from a defective HSV-1 amplicon vector protects pancreatic beta-cells from apoptosis. *Human Gene Ther* 1996;7:1719–1726
- Iwahashi H, Hanafusa T, Eguchi Y, et al. Cytokine-induced apoptotic cell death in a mouse pancreatic beta-cell line: inhibition by Bcl-2. *Diabetologia* 1996;39:530–536
- Rabinovitch A, Suarez-Pinzon W, Strynadka K, et al. Transfection of human pancreatic islets with an anti-apoptotic gene (bcl-2) protects  $\beta$ -cells from cytokine-induced destruction. *Diabetes* 1999;48:1223–1229
- Dupraz P, Rinsch C, Pralong WF, et al. Lentivirus-mediated Bcl-2 expression in betaTC-tet cells improves resistance to hypoxia and cytokine-induced apoptosis while preserving in vitro and in vivo control of insulin secretion. *Gene Ther* 1999;6:1160–1169
- Petersen L, Størling J, Heding P, et al. IL-1 $\beta$ -induced pro-apoptotic signalling is facilitated by NCAM/FGF receptor signalling and inhibited by the C3d ligand in the INS-1E rat beta cell line. *Diabetologia* 2006;49:1864–1875
- Paraskevas S, Aikin R, Maysinger D, et al. Modulation of JNK and p38 stress activated protein kinases in isolated islets of Langerhans: insulin as an autocrine survival signal. *Ann Surg* 2001;233:124–133
- Wang L, Bhattacharjee A, Zuo Z, et al. A low voltage-activated Ca<sup>2+</sup> current mediates cytokine-induced pancreatic beta-cell death. *Endocrinology* 1999;140:1200–1204
- Størling J, Binzer J, Andersson A, et al. Nitric oxide contributes to cytokine-induced apoptosis in pancreatic beta cells via potentiation of JNK activity and inhibition of Akt. *Diabetologia* 2005;48:2039–2050
- Aikin R, Maysinger D, Rosenberg L. Cross-talk between phosphatidylinositol 3-kinase/AKT and c-jun NH2-terminal kinase mediates survival of isolated human islets. *Endocrinology* 2004;145:4522–4531
- Nielsen K, Karlens AE, Deckert M, et al.  $\beta$ -Cell maturation leads to in vitro sensitivity to cytotoxins. *Diabetes* 1999;48:2324–2332
- Gomez JA, Gama V, Yoshida T, et al. Bax-inhibiting peptides derived from Ku70 and cell-penetrating pentapeptides. *Biochem Soc Trans* 2007;35:797–801
- Cohen HY, Lavu S, Bitterman KJ, et al. Acetylation of the C terminus of Ku70 by CBP and PCAF controls Bax-mediated apoptosis. *Mol Cell* 2004;13:627–638
- Subramanian C, Opari AW Jr, Bian X, et al. Ku70 acetylation mediates neuroblastoma cell death induced by histone deacetylase inhibitors. *Proc Natl Acad Sci U S A* 2005;102:4842–4847
- Yoshida T, Tomioka I, Nagahara T, et al. Bax-inhibiting peptide derived from mouse and rat Ku70. *Biochem Biophys Res Commun* 2004;321:961–966
- Maechler P, Wollheim CB. Mitochondrial function in normal and diabetic beta-cells. *Nature* 2001;414:807–812
- Zhou YP, Pena JC, Roe MW, et al. Overexpression of Bcl-xL in beta-cells prevents cell death but impairs mitochondrial signal for insulin secretion. *Am J Physiol Endocrinol Metab* 2000;278:E340–E351
- Doyle ME, Egan JM. Pharmacological agents that directly modulate insulin secretion. *Pharmacol Rev* 2003;55:105–131
- Mao Z, Wiedmann M. Calcineurin enhances MEF2 DNA binding activity in calcium-dependent survival of cerebellar granule neurons. *J Biol Chem* 1999;274:31102–31107
- Matsuda S, Shibasaki F, Takehana K, et al. Two distinct action mechanisms of immunophilin-ligand complexes for the blockade of T-cell activation. *Embo Rep* 2000;1:428–434
- Collier JJ, Fueger PT, Hohmeier HE, et al. Pro- and antiapoptotic cytokines regulate apoptosis but do not protect against cytokine-mediated cytotoxicity in rat islets and  $\beta$ -cell lines. *Diabetes* 2006;55:1398–1406
- Hohmeier HE, Mulder H, Chen G, et al. Isolation of INS-1–derived cell lines with robust ATP-sensitive K<sup>+</sup> channel-dependent and -independent glucose-stimulated insulin secretion. *Diabetes* 2000;49:424–430
- Chen G, Hohmeier HE, Gasa R, et al. Selection of insulinoma cell lines with resistance to interleukin-1 $\beta$ - and  $\gamma$ -interferon-induced cytotoxicity. *Diabetes* 2000;49:562–570
- Cottet S, Dupraz P, Hamburger F, et al. cFLIP protein prevents tumor necrosis factor- $\alpha$ -mediated induction of caspase-8–dependent apoptosis in insulin-secreting  $\beta$ Tc-Tet cells. *Diabetes* 2002;51:1805–1814
- McKenzie MD, Carrington EM, Kaufmann T, et al. Proapoptotic BH3-only protein Bid is essential for death receptor–induced apoptosis of pancreatic  $\beta$ -cells. *Diabetes* 2008;57:1284–1292
- Tunon MJ, Sanchez-Campos S, Gutierrez B, et al. Effects of FK506 and rapamycin on generation of reactive oxygen species, nitric oxide production and nuclear factor kappa B activation in rat hepatocytes. *Biochem Pharmacol* 2003;66:439–445
- Strasser S, Alejandro R, Shapiro ET, et al. Effect of FK506 on insulin secretion in normal dogs. *Metabolism* 1992;41:64–67
- Redmon JB, Olson LK, Armstrong MB, et al. Effects of Tacrolimus (FK506) on human insulin gene expression, insulin mRNA levels, and insulin secretion in HIT-T15 cells. *J Clin Invest* 1996;98:2786–2793
- Polastri L, Galbiati F, Bertuzzi F, et al. Secretory defects induced by immunosuppressive agents on human pancreatic beta-cells. *Acta Diabetologica* 2002;39:229–233
- Drachenberg CB, Klassen DK, Weir MR, et al. Islet cell damage associated with tacrolimus and cyclosporine: morphological features in pancreas allograft biopsies and clinical correlation. *Transplantation (Baltimore)* 1999;68:396–402
- Lawrence MC, Bhatt HS, Watterson JM, et al. Regulation of insulin gene transcription by a Ca<sup>2+</sup>-responsive pathway involving calcineurin and nuclear factor of activated T cells. *Mol Endocrinol* 2001;15:1758–1767
- Aikin R, Hanley S, Maysinger D, et al. Autocrine insulin action activates Akt and increases survival of isolated human islets. *Diabetologia* 2006;49:2900–2909