*Original Article*

# Pro- and Antiapoptotic Proteins Regulate Apoptosis but Do Not Protect Against Cytokine-Mediated Cytotoxicity in Rat Islets and β-Cell Lines

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**Type 1 diabetes results from islet β-cell death and dysfunction induced by an autoimmune mechanism. Proinflammatory cytokines such as interleukin-1β and γ-interferon are mediators of this β-cell cytotoxicity, but the mechanism by which damage occurs is not well understood. In the current study, we present multiple lines of evidence supporting the conclusion that cytokine-induced killing of rat β-cells occurs predominantly by a nonapoptotic mechanism, including the following: *1*) A rat β-cell line selected for resistance to cytokine-induced cytotoxicity (833/15) is equally sensitive to killing by the apoptosis-inducing agents camptothecin and etoposide as a cytokine-sensitive cell line (832/13). *2*) Overexpression of a constitutively active form of the antiapoptotic protein kinase Akt1 in 832/13 cells provides significant protection against cell killing induced by camptothecin and etoposide but no protection against cytokine-mediated damage. *3*) Small interfering RNA–mediated suppression of the proapoptotic protein Bax enhances viability of 832/13 cells upon exposure to the known apoptosis-inducing drugs but not the inflammatory cytokines. *4*) Exposure of primary rat islets or 832/13 cells to the inflammatory cytokines causes cell death as evidenced by the release of adenylate kinase activity into the cell medium, with no attendant increase in caspase 3 activation or annexin V staining. In contrast, camptothecin- and etoposide-induced killing is associated with robust increases in caspase 3 activation and annexin V staining. *5*) Camptothecin increases cellular ATP levels, whereas inflammatory cytokines lower ATP levels in both β-cell lines and primary islets. We conclude that proinflammatory cytokines cause β-cell cytotoxicity primarily through a nonapoptotic mechanism linked to a decline in ATP levels. *Diabetes* 55:1398–1406, 2006**

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Received for publication 4 August 2005 and accepted in revised form 30 January 2006.

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γ-IFN, γ-interferon; IL, interleukin; iNOS, inducible nitric oxide synthase; siRNA, small interfering RNA.

DOI: 10.2337/db05-1000

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Type 1 diabetes is an autoimmune disease that results from selective destruction of pancreatic β-cells within the islets of Langerhans. Damage to the insulin-producing β-cells occurs via T-cell and macrophage infiltration of the islet and exposure to proinflammatory cytokines that these cells produce, such as interleukin (IL)-1β and γ-interferon (γ-IFN). The mechanism underlying the cytotoxic effects of the inflammatory cytokines is not completely understood. A role for inducible nitric oxide synthase (iNOS) and NO production in IL-1β– and γ-IFN–mediated β-cell death and dysfunction has been clearly established (1,2). However, it remains uncertain whether IL-1β and γ-IFN induce an apoptotic death program, an autophagic cell death pathway, a cell lysis/necrosis pathway, or some combination of these events (3–5).

Apoptosis is dependent upon the activation of caspases (6). Caspases are synthesized as catalytically inactive zymogens and must undergo proteolytic activation during apoptosis. Initiator caspases, such as caspases 8 and 9, are required to process the executioner caspases, such as caspase 3, leading to their activation. Active caspase 3 cleaves over 40 intracellular substrates that cause cell death (7). It has been suggested that IL-1β and γ-IFN induce apoptosis in islet β-cells, although the extent to which this occurs is controversial (3–5,8). Moreover, confusion exists regarding the role of caspase 3 during cytokine-mediated β-cell death (9–12). The role of nonapoptotic cell death pathways such as necrosis or autophagy is even less well defined and more difficult to quantify experimentally (rev. in 13,14). In the current study, we reasoned that if apoptosis represents the predominant mechanism underlying the loss of β-cell mass during exposure to IL-1β and γ-IFN, manipulation of various pro- and antiapoptotic proteins should protect against cytokine damage.

To test this hypothesis, we have used several strategies to impair apoptosis in β-cell lines and primary rat islets, including *1*) overexpression of a constitutively active form of Akt1, a kinase with antiapoptotic properties, and *2*) decreasing the levels of the proapoptotic proteins Bak and Bax, either alone or in concert, via small interfering RNA (siRNA) duplexes. We find that these maneuvers clearly increase viability of β-cells exposed to classical apoptosis-inducing agents such as camptothecin and etoposide but are unable to protect against the cytotoxic effects of IL-1β and γ-IFN. Moreover, we show that the apoptosis-inducing drugs cause caspase 3 activation, increase annexin V

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staining, and increase cellular ATP levels, whereas inflammatory cytokines cause no significant increases in caspase 3 activity or annexin V staining and decrease intracellular ATP levels in β-cell lines and primary rat islets.

## RESEARCH DESIGN AND METHODS

**Cell culture, adenoviral constructs, nitrite, and viability assays.** The isolation, characterization, and cell culture conditions for the INS-1–derived 832/13 (15) and 833/15 (16) rat insulinoma cell lines were previously described. The myrAkt1 adenovirus used in this study was a gift from Dr. Ken Walsh (Boston University) and was previously described (17). Nitrite in the cell culture media was measured as an index of NO production using the Griess assay kit (Promega, Madison, WI), according to the manufacturer's protocol. Cell viability was determined using the MTS 1 solution kit (Promega), according to the manufacturer's recommendation, or measurement of adenylate kinase activity in the culture medium (BioVision, Mountain View, CA), an index of cell lysis. Pancreatic islets of Langerhans were isolated from ~250-g male Wistar rats by a previously described procedure (18,19) approved by the Duke University Institutional Animal Care and Use Committee.

**siRNA-mediated suppression of gene expression.** For siRNA-directed suppression of Bak and Bax expression, preannealed duplexes were obtained from Ambion (identification nos. 49750 and 190465, respectively; Austin, TX) and transfected into 832/13 cells via the Amaxa nucleofection system (Amaxa, Gaithersburg, MD) using 2 μg of duplex per $2 \times 10^6$ cells in T-solution. The cells were seeded into six-well plates, and suppression of the targeted genes was monitored by immunoblotting.

**Isolation of RNA and real-time detection of mRNA by RT-PCR.** RNA was isolated and purified using TRIzol (Invitrogen, Carlsbad, CA). First-strand cDNA synthesis was performed with 0.5 μg of RNA using iScript (BioRad, Hercules, CA) reagents. Real-time PCR–based measurements of mRNA levels were performed as previously described (20) using TaqMan primer/probe sets and the manufacturer's recommended methodology (Rn0058364 for Akt1 and Rn00567290 for Akt2; Applied Biosystems, Foster City, CA).

**Isolation of protein and immunoblotting.** Cells were seeded into six-well dishes for most of the experiments performed in this study. Whole-cell lysates were prepared using M-PER lysis buffer (Pierce, Rockford, IL) supplemented with protease (Pierce) and phosphatase inhibitor cocktails (Sigma, St. Louis, MO), and 200 μl of lysis buffer were used per well. The protein concentration of the lysate was determined using the bicinchoninic acid assay (Pierce) using BSA as the standard. A total of 20 μg of whole-cell lysate were separated on either 4–12% or 10% Bis-Tris SDS-PAGE gels (Invitrogen, Carlsbad, CA), followed by electrophoretic transfer to polyvinylidine fluoride membranes. The membranes were blocked with 1 × milk buffer (Chemicon, Temecula, CA) for 15 min. Primary antibodies, which were diluted in polyvinylpyrrolidone as described (21), were incubated with the membranes overnight at 4°C. The membranes were washed three times for 5 min each time with Tris-buffered saline. Secondary antibodies were diluted in 1 × milk buffer (Chemicon) and incubated at room temperature for 45 min, followed by washing in Tris-buffered saline three times for 10 min each time. The membranes were then exposed to chemiluminescent substrate and images taken using the VersaDoc imaging system (BioRad). Antibodies that detect Akt, PO4-Akt, and caspase 3 (full-length and cleaved) were from Cell Signaling (Beverly, MA). Anti-Bak and Bax antibodies were from Upstate (Charlottesville, VA). Anti-tubulin (control for protein loading) antibody was from Sigma.

**Caspase 3 activity and intracellular ATP assays.** Whole-cell lysates were prepared using radioimmunoprecipitation assay buffer without protease inhibitors and analyzed for caspase 3 enzyme activity using a kit from Upstate and a Spectramax Gemini XS (Molecular Devices, Sunnyvale, CA) fluorescent plate reader. For measurements of ATP levels, cells were lysed in nucleotide-releasing buffer (Calbiochem, San Diego, CA) and luciferase activity monitored according to the manufacturer's recommendations.

**Annexin V–fluorescein isothiocyanate staining.** Cells were removed from plates by scraping and washed in PBS. The externalization of phosphatidylserine was detected by hybridization to fluorescein isothiocyanate–conjugated annexin V, according to the manufacturer's recommendations (Trevigen, Gaithersburg, MD), and quantified by flow cytometry.

**Statistical analyses.** A one-way ANOVA was performed to detect statistical differences ($P < 0.05$). Differences within the ANOVA were determined using a Tukey post hoc test. All data are reported as means ± SE.

## RESULTS

**Differential effects of cytokines and apoptosis-inducing drugs in cytokine-resistant and -sensitive β-cell lines.** We have previously described cytokine-resistant INS-1–derived β-cell lines, developed via a selection strategy involving long-term culture in increasing doses of IL-1β plus γ-IFN (16,22). We compared the effects of cytokines with those of camptothecin and etoposide, topoisomerase I and II inhibitors and known inducers of apoptosis (21,23), in a cell line selected for cytokine resistance (line 833/15) versus a cell line that did not undergo selection (line 832/13). As shown in Fig. 1, 24 h of exposure to 10 ng/ml IL-1β and 100 units/ml γ-IFN resulted in a 40% decrease in cell viability as measured by MTS assay in 832/13 cells but no killing of 833/15 cells. In contrast, exposure of either cell line to camptothecin plus etoposide for 24 h resulted in an identical 80% decrease in cell viability. Thus, selection for cytokine resistance provides complete protection against cytokine-induced cell killing but no protection against apoptosis-inducing drugs.

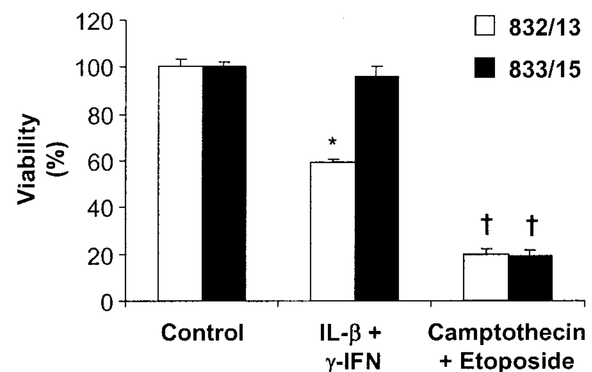


**FIG. 1. Differential responses of INS-1–derived cell lines to proinflammatory cytokines and apoptosis-inducing agents. Cytokine-sensitive (832/13) or -resistant (833/15) rat INS-1–derived cell lines were exposed to media alone (Control) or media containing 10 ng/ml IL-1β and 100 units/ml γ-IFN or 2 μmol/l camptothecin plus 100 μmol/l etoposide for 24 h. Cell viability was measured by the MTS assay. Data are means ± SE for three independent experiments. *$P < 0.01$ vs. control and 833/15; †$P < 0.01$ vs. control and IL-1β + γ-IFN.**

**Camptothecin- and etoposide-mediated decreases in viability of cytokine-sensitive 832/13 cells and cytokine-resistant 833/15 cells is partially corrected by Akt1 overexpression.** The antiapoptotic serine/threonine kinase Akt is well documented as a key regulator of cell viability (23). Adenovirus-mediated overexpression of constitutively active Akt1 in the 832/13 rat insulinoma cell line lead to a fourfold increase in Akt1 mRNA levels (Fig. 2*A*), correlating with increases in both total (fourfold) and phosphorylated (eightfold) Akt protein levels (Fig. 2*B*). Similar levels of Akt1 overexpression were achieved in 833/15 cells (data not shown). Expression of the constitutively active Akt1 in either cell line nearly doubled viability relative to AdCMV-green fluorescent protein–treated and no virus controls in cells exposed to camptothecin (Fig. 2*C*).

**siRNA-mediated suppression of Bax expression partially prevents camptothecin-mediated decreases in 832/13 cell viability.** The proapoptotic proteins Bak and Bax induce apoptosis by interacting with various cellular proteins via their BH3 domains and perhaps by promoting mitochondrial dysfunction, although the precise mechanism is unclear (24). These proteins are present in the rat islet (25,26), and we therefore hypothesized that suppression of their expression via delivery of siRNA duplexes would protect β-cells against apoptosis. siRNA duplexes

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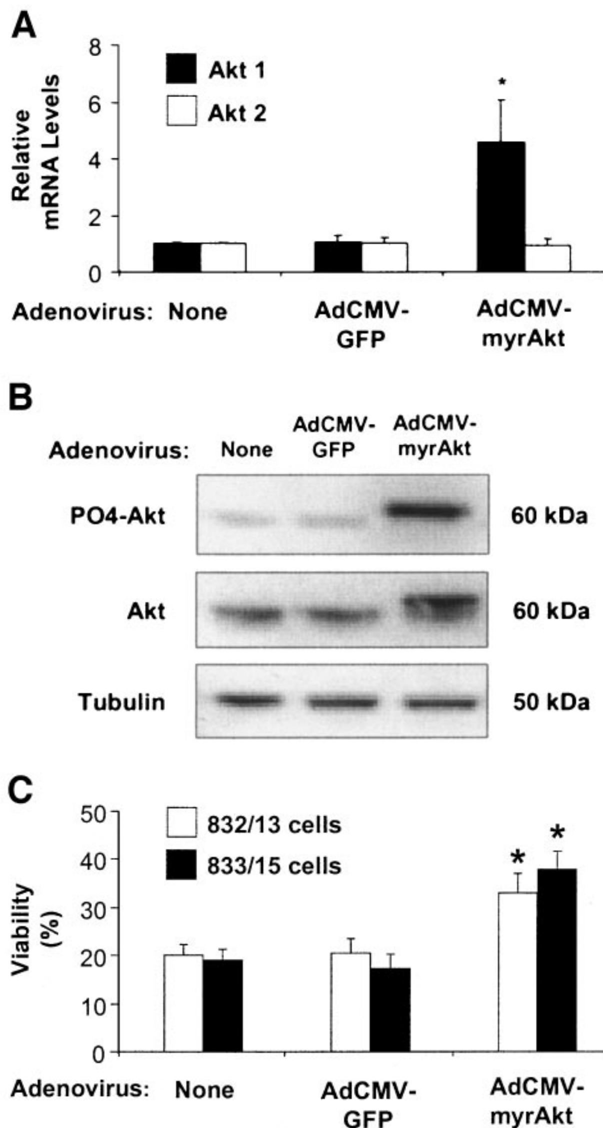


**FIG. 2. Overexpression of constitutively active Akt1 partially protects against camptothecin/etoposide-induced apoptosis in both 832/13 and 833/15 INS-1 cells. *A* and *B*: 832/13 cells were transduced with the indicated recombinant adenoviruses, followed by culture of the cells for 40 h and harvesting of whole-cell lysates. The levels of Akt1 and Akt2 mRNA were determined by quantitative RT-PCR (*A*) and total Akt (Akt) and phosphorylated Akt (PO4-Akt), and tubulin protein levels were determined by immunoblotting (*B*). The ectopically expressed constitutively active form of Akt1 migrates slightly slower than the endogenous protein due to the addition of a myristoylation signal and HA-epitope tagging of the former. *C*: 832/13 and 833/15 cells were transduced with the indicated adenoviruses, cultured overnight, and treated with 2 μmol/l camptothecin plus 100 μmol/l etoposide to induce apoptosis for the final 16 h of the 40 h following adenoviral transduction. Cell viability was determined by the MTS assay. Data are expressed relative to cells receiving no virus and no additional effectors (not shown), which were considered to be 100% viable and are means ± SE for three independent experiments. *$P < 0.01$ vs. other groups. GFP, green fluorescent protein.**

specific for Bax and Bak were individually transfected or transfected in combination into 832/13 cells, resulting in 86 ± 3 and 84 ± 4% decreases in the levels of these proteins, respectively (Fig. 3*A*). This amount of siRNA-mediated suppression of Bax expression resulted in an increase in cell viability from 27 to 49% during 24-h exposure to camptothecin (Fig. 3*B*). Interestingly, silencing of Bak alone did not confer protection against camptothecin, and concurrent silencing of Bax and Bak gave the same level of protection as did silencing of Bax alone.

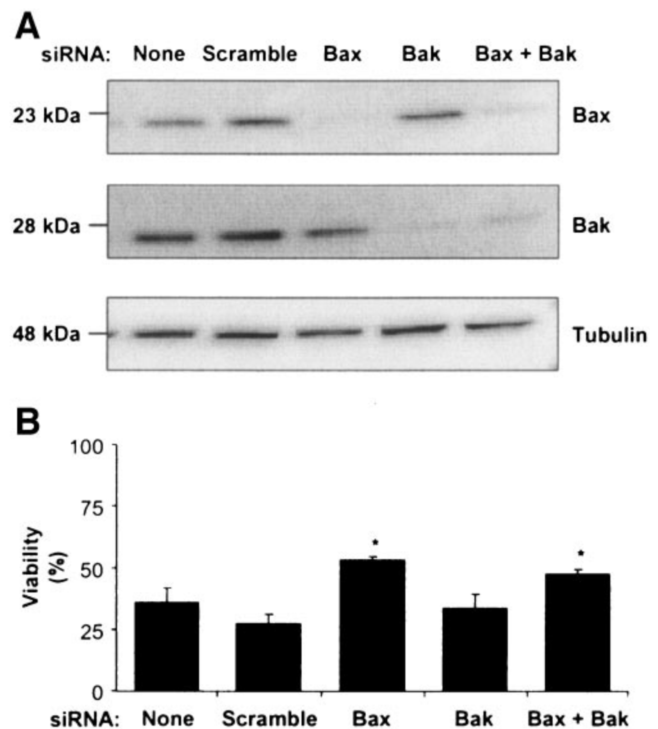


**FIG. 3. siRNA-mediated suppression of Bax, but not Bak, expression partially protects against apoptosis. The proapoptotic proteins Bax and Bak were silenced independently or in combination by RNA interference in 832/13 INS-1 cells. *A*: Shown is a representative immunoblot for Bax, Bak, and the loading control protein tubulin. *B*: 832/13 INS-1 cells were transfected with siRNAs directed against Bax and Bak or a scrambled control siRNA (Scramble). When cells were >80% confluent (48 h following transfection), they were exposed to 2 μmol/l camptothecin for 24 h. Cell viability was measured by the MTS assay. Cell viability data are normalized to cells that were not treated with camptothecin. Data are means ± SE for four independent experiments. *$P < 0.05$ vs. None, Scramble, and Bak.**

**Camptothecin or etoposide, but not IL-1β and γ-IFN, induces caspase 3 cleavage and enzyme activity, an effect blunted by expression of constitutively active Akt1 or silencing of Bax.** The presence of cleaved caspase 3 or an increase in caspase 3 enzymatic activity are often used as markers of the apoptotic signaling cascade (27). Exposure of 832/13 cells to camptothecin plus etoposide for 6 h stimulated caspase 3 cleavage (Fig. 4*A*). Stimulation of caspase 3 cleavage by camptothecin plus etoposide treatment was suppressed by 58% in response to overexpression of constitutively active Akt1 (Fig. 4*A*). Camptothecin or etoposide treatment also increased caspase 3 enzymatic activity by two- to threefold, an effect blunted by Akt1 overexpression (60 and 70%, respectively) (Fig. 4*B*). In contrast, treatment of 832/13 cells with IL-1β plus γ-IFN for 6 h (data not shown) or 24 h (Fig. 4*B*) had no effect on caspase 3 activity. Silencing of the proapoptotic protein Bax also decreased camptothecin-mediated caspase 3 enzyme activity (Fig. 4*C*). We conclude that caspase 3 cleavage, a mediator of the apoptotic cascade, is suppressed by overexpression of constitutively active Akt1 or by silencing of Bax in INS-1–derived cell lines, thereby potentially explaining the cytoprotective effects of these agents documented in Figs. 2 and 3. In contrast, the cytotoxicity elicited by incubation of the same cells with inflammatory cytokines is not associated with activation of caspase 3.

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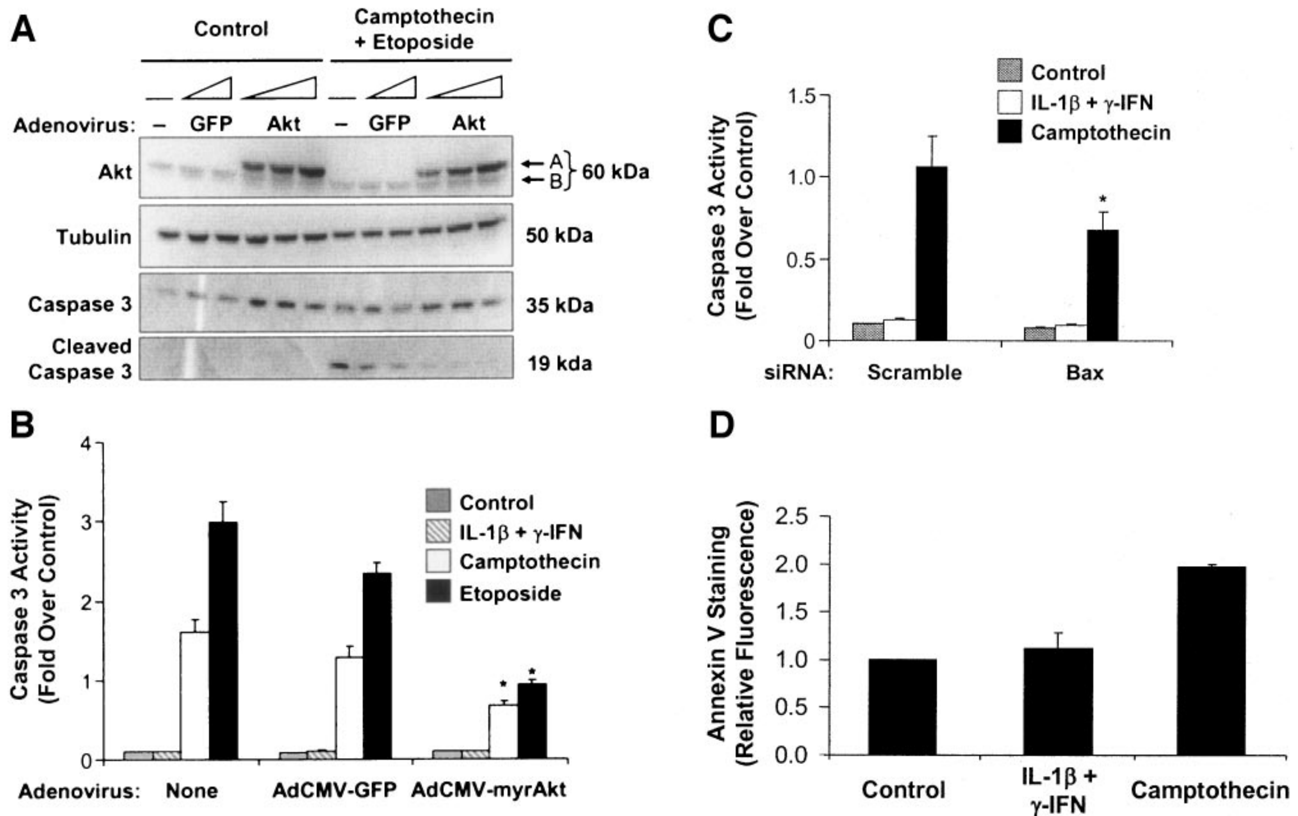


**FIG. 4. Caspase 3 cleavage and enzyme activity are regulated by apoptosis-inducing agents but not proinflammatory cytokines. 832/13 INS-1 cells were transduced with increasing doses of recombinant adenoviruses as in Fig. 2 and treated with media alone (Control) or media containing 2 μmol/l camptothecin and 100 μmol/l etoposide for 6 h. *A*: Whole-cell extracts were then immunoblotted for total Akt, total caspase 3, cleaved caspase 3, and tubulin. Note that the ectopically expressed form of Akt is identified as *A* and the endogenous form as *B*. *B* and *C*: Caspase 3 activity was measured in 832/13 INS-1 cells transduced with recombinant adenoviruses as in Fig. 2 (*B*) or transfected with siRNA duplexes as in Fig. 3 (*C*) and treated with media alone for 24 h (Control) or that containing 10 ng/ml IL-1β and 100 units/ml γ-IFN for 24 h, 2 μmol/l camptothecin for 6 h, or 100 μmol/l etoposide for 6 h. Data are means ± SE for three independent experiments. **P* < 0.05 vs. None and AdCMV-green fluorescent protein (GFP) for adenovirus experiments and scramble for siRNA experiments. *D*: 832/13 INS-1 cells were treated with media alone for 24 h (Control) or that containing 10 ng/ml IL-1β and 100 units/ml γ-IFN for 24 h or 2 μmol/l camptothecin for 6 h. Annexin V–fluorescein isothiocyanate staining was measured by flow cytometry, and data are presented as the mean of two independent experiments.**

As an independent measure of apoptosis, annexin V staining was performed in cells exposed to cytokines or camptothecin. Early in apoptosis, phosphotidylserine flips in the plasma membrane, thereby exposing it to the exterior of the cell and permitting annexin V binding. As shown in Fig. 4*D*, camptothecin caused a doubling of annexin V–fluorescein isothiocyanate staining in 832/13 cells, whereas cytokines had no significant effect, consistent with the caspase 3 activation assays.

**Overexpression of Akt1 does not protect 832/13 rat insulinoma cells from the combined effects of IL-1β and γ-IFN.** Exposure of the cytokine-sensitive 832/13 cells to 10 ng/ml IL-1β and 100 units/ml γ-IFN for 24 h decreased cell viability by ~40%, as measured by the MTS assay (Figs. 1 and 5). Overexpression of constitutively active Akt1 (17), which partially protected against apoptosis (Fig. 2*C*), had no effect on IL-1β– and γ-IFN–mediated increases in nitrite production (Fig. 5*A*) or cell killing (Fig. 5*B*). Phospho-Akt levels were not diminished by treatment with IL-1β and γ-IFN (Fig. 5*C*), removing cytokine-mediated dephosphorylation of Akt1 as a potential explanation for the lack of protective effect of Akt overexpression. Taken together with the lack of caspase 3 activation by IL-1β and γ-IFN (Fig. 4*B* and *C*), we conclude that apoptosis is not a major pathway of cytokine-induced death in 832/13 cells.

The MTS viability assay measures metabolism of a tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) in functional mitochondria. To obtain an independent measure of cell killing by cytokines and apoptosis-inducing agents, we measured adenylate kinase enzymatic activity in tissue culture medium of cells exposed to these agents. Following 24 h of exposure, all of the cytotoxic agents tested (cytokines, camptothecin, etoposide, and, as a positive control, the β-cell cytotoxin streptozotocin) induced a six- to sevenfold increase in medium adenylate kinase activity, indicative of a loss in cellular membrane integrity and release of intracellular stores of the enzyme (Table 1). These data confirm that both cytokines and the apoptotic agents induce bona fide cell killing.

**Silencing of Bak and Bax does not prevent IL-1β– and γ-IFN–induced decreases in 832/13 insulinoma cell viability.** To validate the foregoing results by an independent approach, we silenced the proapoptotic proteins Bax and Bak to determine whether this manuever prevents cytokine-mediated cytotoxicity. Transfection of siRNA duplexes that target transcripts of either Bak or Bax into 832/13 cells led to a marked decrease in the levels of each of these proteins (Fig. 3*A*). However, this silencing failed to prevent the IL-1β– and γ-IFN–mediated stimulation of

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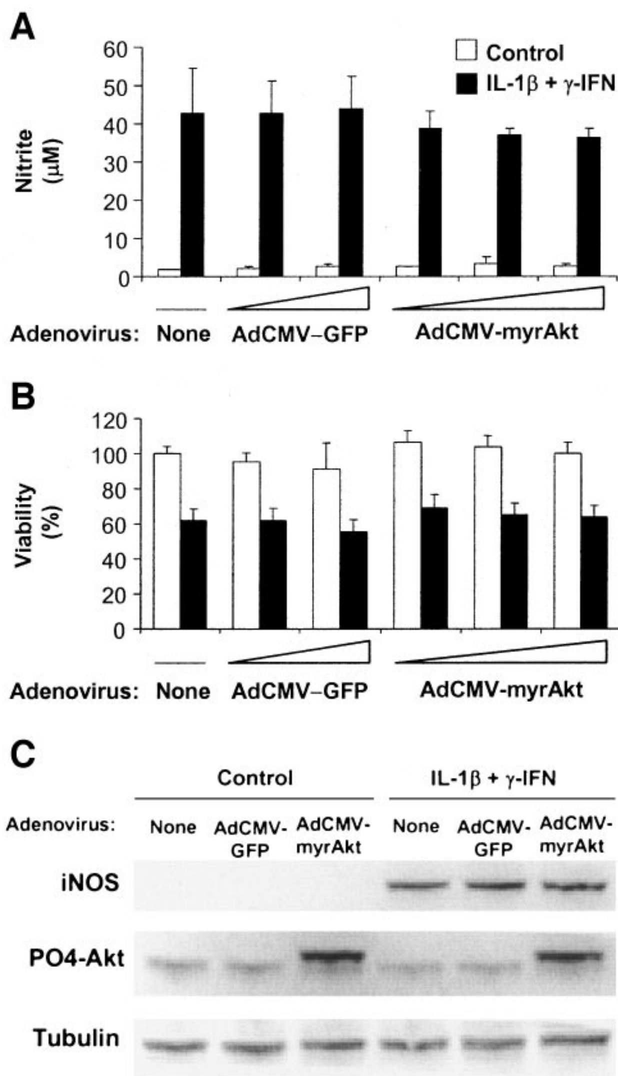


**FIG. 5. Overexpression of constitutively active Akt1 does not prevent IL-1β– and γ-IFN–induced nitrite production or decreases in cell viability. 832/13 INS-1 cells were transduced with increasing doses of recombinant adenoviruses as in Fig. 2. When cells were 80% confluent (48 h following transfection), they were exposed to media alone (Control) or media containing 10 ng/ml IL-1β and 100 units/ml γ-IFN for 24 h. *A*: Nitrite was measured in the media by the Griess method following the 24-h treatment period. *B*: Cell viability was measured by the MTS assay. Cell viability data are normalized to cells exposed to media alone. Data are means ± SE for four independent experiments. No statistical differences were detected between groups. *C*: 832/13 cells were transduced with the indicated recombinant adenoviruses, cultured for 40 h, and treated with media alone (Control) or that containing 10 ng/ml IL-1β and 100 units/ml γ-IFN for the final 16 h. Whole-cell lysates were immunoblotted for total Akt (Akt), phosphorylated Akt (PO4-Akt), and tubulin. GFP, green fluorescent protein.**

iNOS (Fig. 6*A*) or nitrite production (Fig. 6*B*) or the cytotoxic effects of the cytokines (Fig. 6*C*).

**Camptothecin, but not IL-1β and γ-IFN, induces caspase 3 enzyme activity in isolated rat islets.** We next sought to verify that the results obtained in insulinoma cell lines are reproducible in primary cells. In isolated rat islets, we found that 6 (data not shown)- or 24-h exposure to 10 ng/ml IL-1β and 100 units/ml γ-IFN caused no significant increase in caspase 3 enzyme activity, whereas exposure to camptothecin for 6 h caused a 17-fold increase in enzyme activity (Fig. 7*A*). However, treatment with the proinflammatory cytokines did induce a robust increase in medium adenylate kinase, indicative of cell death by membrane rupture (Fig. 7*B*). These data show that although IL-1β and γ-IFN are clearly cytotoxic to primary islets, these effects are mediated predominantly by caspase 3–independent nonapoptotic pathways, in complete agreement with our observations in insulinoma cell lines.

TABLE 1
Comparison of cell viability and lysis measurements in 832/13 cells

| | Viability by MTS assay (%) | Adenylate kinase assay (fold) |
|---|---|---|
| Control | 100 ± 6 | 1.0 ± 0.3 |
| IL-1β and γ-IFN | 60 ± 1* | 6.6 ± 1.1* |
| Camptothecin | 42 ± 4† | 7.1 ± 0.7* |
| Etoposide | 52 ± 3† | 7.4 ± 1.0* |
| Streptozotocin | 49 ± 4† | 7.5 ± 0.9* |

Data are means ± SE. Data were normalized to control cells and expressed as percent for the MTS assay or fold increase for the adenylate kinase assay. Cytokine-sensitive 832/13 cells were treated with medium alone (Control) or media containing 10 ng/ml IL-1β and 100 units/ml γ-IFN, 2 μmol/l camptothecin, 100 μmol/l etoposide, or 1 mmol/l streptozotocin for 24 h. Cell viability was measured by the MTS assay, and cell lysis was measured by the presence of adenylate kinase activity in the media. $^*P < 0.05$ vs. control; $^†P < 0.05$ vs. control and IL-1β and γ-IFN.

**Camptothecin and cytokines differentially impact intracellular ATP levels in both rat insulinoma cells and isolated rat islets.** Activation of caspases requires energy, and it is therefore presumed that apoptosis requires relatively high intracellular ATP levels. By contrast, depletion of ATP has been proposed to divert cells toward other death routes (14). In 832/13 cells, 833/15 cells, and isolated rat islets, camptothecin increased ATP levels, consistent with its ability to induce apoptosis killing in all three cell populations (Fig. 8). In contrast, IL-1β and γ-IFN caused a decrease in the concentration of the nucleotide in cytokine-sensitive 832/13 cells and primary rat islets but failed to induce this decrease in cytokine-resistant 833/15 cells (Fig. 8). Our results are consistent with a previous report of effects of proinflammatory cytokines to decrease β-cell ATP levels (28). However, both the lack of effect of cytokines on ATP levels in cytokine-resistant cell lines and the effect of camptothecin to increase ATP levels in β-cells are novel observations. These results further support the concept that inflammatory cytokines cause cell death primarily by a mechanism independent of that employed by classical inducers of apoptosis.

## DISCUSSION

Type 1 diabetes is caused by islet β-cell destruction associated with infiltration of the pancreatic islets by T-cells and macrophages (insulitis) and local production of the proinflammatory cytokines IL-1β and γ-IFN (29,30). While cytokines appear to have a direct role in inducing pancreatic β-cell death, the mechanism by which this is achieved is controversial (3,5,31). On the one hand, there is broad agreement that IL-1β and γ-IFN stimulate iNOS expression and NO production, leading to a decrease in β-cell viability (2). On the other hand, various groups have reported that this loss of viability occurs via activation of apoptosis (31–35), necrosis (3–5,8), or undefined (36–38) form(s) of cell death.

In the current study, we present multiple lines of evidence supporting the conclusion that cytokine-induced

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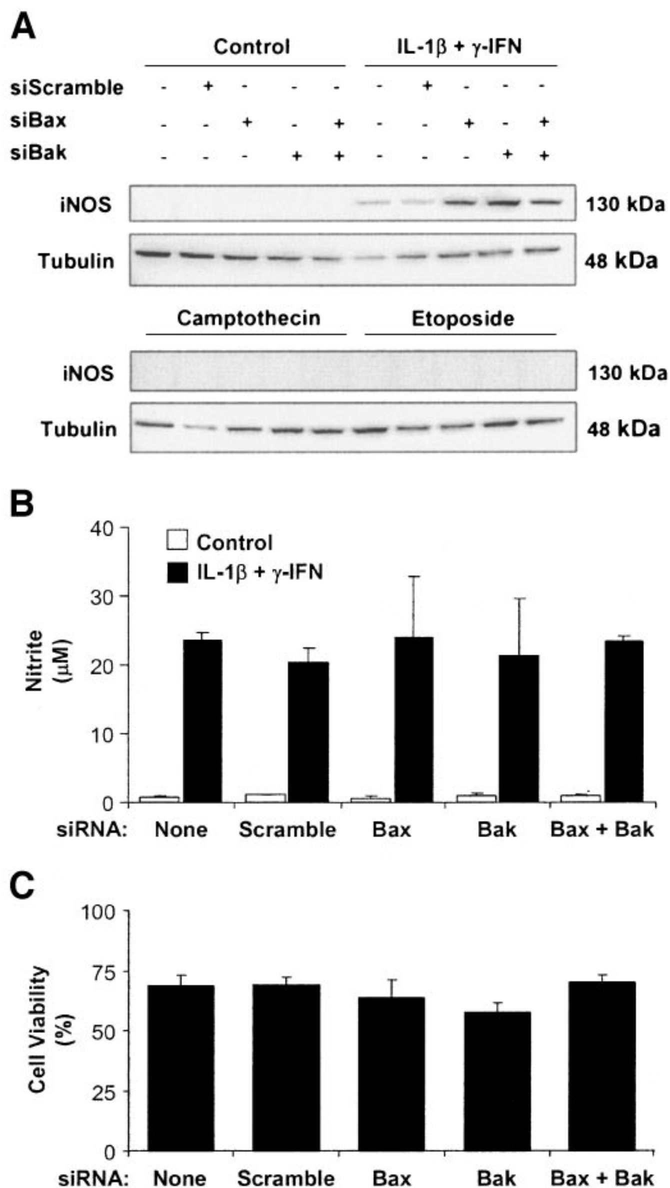


FIG. 6. siRNA-mediated suppression of Bak and Bax expression does not prevent IL-1β– and γ-IFN–induced nitrite production or decreases in cell viability. 832/13 INS-1 cells were transfected with siRNAs directed against Bax and Bak or a scrambled control siRNA. When cells were 80% confluent, they were exposed to media (Control) or 10 ng/ml IL-1β and 100 units/ml γ-IFN for 24 h. *A*: Whole-cell extracts were immunoblotted for iNOS. Tubulin served as a loading control. The blots are representative of two independent experiments. *B*: Nitrite was measured in the media by the Griess method following the 24-h treatment period. *C*: Cell viability was measured by the MTS assay. Cell viability data are normalized to cells exposed to media alone. Data are means ± SE.

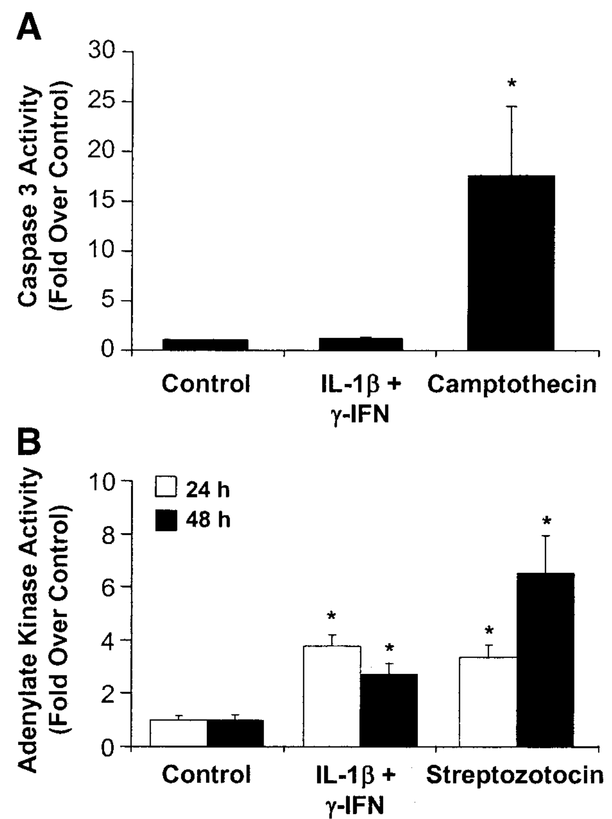


FIG. 7. Camptothecin, but not IL-1β and γ-IFN, induces caspase 3 enzyme activity in rat islets. *A*: Primary rat islets were isolated and treated with media alone (Control) for 24 h or media containing either 10 ng/ml IL-1β and 100 units/ml γ-IFN for 24 h or 2 μmol/l camptothecin for 6 h. Caspase 3 enzyme activity was then measured. Data are means ± SE for three independent experiments. $^*P < 0.01$ vs. other groups. *B*: Primary rat islets were isolated and treated with media alone (Control) or media containing either 10 ng/ml IL-1β and 100 units/ml γ-IFN or 1 mmol/l streptozotocin. Adenylate kinase activity was measured in the media after 24 and 48 h. Data are normalized to control islets and are means ± SE for three independent experiments, performed in duplicate each time. $^*P < 0.05$ vs. other groups.

killing of rat β-cells occurs predominantly by a nonapoptotic mechanism, including the following: *1*) A cell line selected for resistance to cytokine-induced cytotoxicity (833/15) is equally sensitive to killing by the apoptosis-inducing agents camptothecin and etoposide as a cytokine-sensitive cell line (832/13) that was not subjected to the selection procedure. *2*) Overexpression of a constitutively active form of Akt1, a known antiapoptotic protein kinase, in 832/13 cells provides significant protection against cell killing induced by camptothecin and etoposide but no protection against cytokine-mediated damage. *3*) siRNA-mediated suppression of the proapoptotic protein Bax enhances viability of 832/13 cells upon exposure to the known apoptosis-inducing drugs but not the inflammatory cytokines. *4*) The apoptosis-inducing drugs, but not cytokines, cause clear increases in caspase 3 cleavage and enzymatic activity in both β-cell lines and primary islets. Caspase 3 activity has recently been described as the most prominent marker for distinguishing apoptotic from other forms of cell death (39). *5*) Camptothecin, but not the inflammatory cytokines, causes a significant increase in annexin V staining, an independent marker of apoptotic cell death. *6*) The apoptosis-inducing drug camptothecin increases cellular ATP levels, as would be expected for pathways requiring caspase activation, whereas inflammatory cytokines lower ATP levels in cytokine-sensitive β-cell lines and primary islets.

Our data arguing for a nonapoptotic mechanism of cytokine-mediated β-cell killing differ from some prior studies (31–35) that claim an important contribution of apoptosis. One possible concern is that the INS-1–derived cell lines used here might be generally refractory to programmed cell death. However, this is clearly not the case, as demonstrated by similarly strong induction of caspase 3 cleavage and enzymatic activity in response to camptothecin treatment in 832/13 cells and primary rat islets. Other differences in experimental design that could

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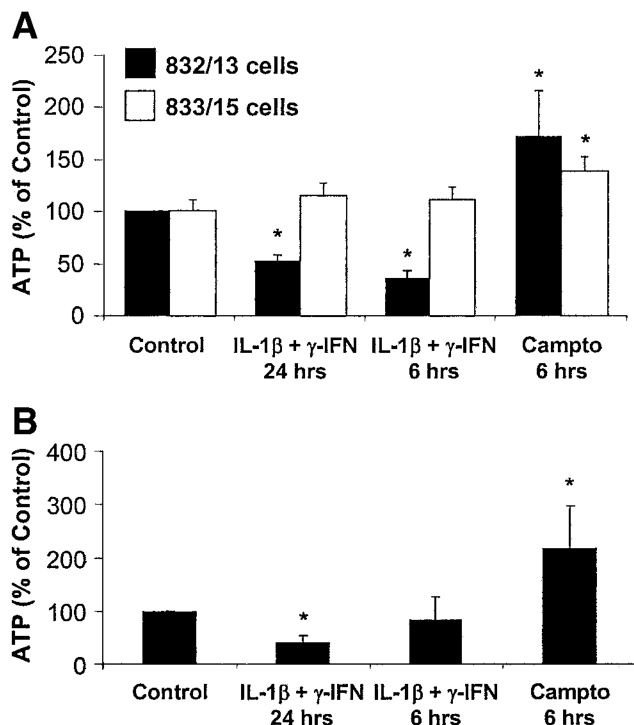


**FIG. 8. Effects of camptothecin and cytokines on intracellular ATP levels in insulinoma cell lines and isolated rat islets. 832/13 and 833/15 cells (*A*) or rat islets (*B*) were treated with either IL-1β and γ-IFN for 6 or 24 h or camptothecin (Campto) for 6 h. Shown are means ± SE for two (islets) or three (832/13, 833/15 cells) independent experiments, each performed in triplicate. *$P < 0.05$ vs. controls.**

have contributed to divergent outcomes include the fact that the present studies were conducted over a period of 6–24 h and were focused on manipulations of anti- and proapoptotic genes, whereas some of the earlier studies involved 6–9 days of cytokine exposure and mainly addressed nuclear factor-κB and iNOS manipulations (4,32). However, other extant studies (34,35) in which apoptotic effects of cytokines were reported were carried out over a similar time frame (18–24 h) as the current study. In one such study (34), cytokines caused a modest increase in the number of apoptotic INS-1E cells from ~2 to 4.5% of the total but, consistent with the findings reported here, caused no significant increase in apoptosis in primary rat islets. Moreover, a bona fide apoptosis-activating agent such as camptothecin was not included as a positive control in the prior study. We suspect that use of an agent such as camptothecin would have revealed a much larger percentage of apoptotic cells than achieved with cytokines, consistent with the data shown herein. Finally, the idea that cytokines would activate caspase 3 seems counterintuitive in the sense that cytokines are well known to stimulate iNOS expression and NO production (Fig. 6*B*), and NO is known to directly inhibit caspase 3 activity (46–49). Therefore, even if cytokines were to cause significant caspase 3 cleavage (which we do not observe), enzyme activity would be inhibited by NO, preventing any pronounced stimulation of apoptosis, which is consistent with our findings and those laboratories that have shown minimal apoptosis in β-cells exposed to IL-1β and γ-IFN (3,8,11,40–42).

Nonapoptotic pathways of cell death could include necrosis, paraptosis, and autophagy (13,39). These forms of cell death are less well defined than apoptosis in terms of biochemical and morphological markers, and the current studies do not clearly define which of the alternative pathways become activated by inflammatory cytokines. However, it has recently been suggested that intracellular ATP levels may be a determinant of the switch from apoptosis to necrosis (14,43). This switch may be mediated by the decrease in ATP levels occurring in islet β-cells upon exposure to cytokines (28; Fig. 7), which in turn may be explained in part by the ability of IL-1β to suppress mitochondrial aconitase activity (44,45), leading to a reduced capacity for apoptotic cell death, an energy-expensive process. Consistent with this idea, the cytokine-mediated fall in ATP levels seen in cytokine-sensitive 832/13 cells and primary rat islets is not observed in cytokine-resistant 833/15 cells. The cellular decision to proceed toward necrotic as opposed to apoptotic death (42) may also depend on NO production in concert with ATP depletion (14). By this view, inflammatory cytokines, particularly IL-1β, which stimulate iNOS expression and generate high levels of NO (44), would be expected to push a cell toward necrotic cell death pathways (14). This idea is supported by studies in which targeted disruption of the iNOS gene in islets decreased the amount of necrotic cell death following exposure to cytokines (4). Moreover, accumulation of NO leads to nitrosylation and inhibition of caspase 3 in a number of different cell types, potentially preventing this cell death pathway (46–49).

Other findings support the distinction between apoptosis-inducing agents and nonapoptotic cell death, as apparently caused by cytokine exposure. Thus, apoptosis is a very rapid process, with full activation of the death pathway occurring within hours of exposure to apoptosis-inducing agents (50). One form of apoptosis in insulin-producing cells is independent of nuclear factor-κB–mediated transcription but dependent on caspase 3 activity and poly(ADP-ribose) polymerase-1 cleavage (51). Cytokine-induced β-cell killing, in contrast, requires the expression of several genes (e.g., iNOS, COX2, etc.), is dependent on nuclear factor-κB activity, and generally occurs over a period of 1 to several days (16,32,36,44).

Some basal level of apoptosis is likely to occur in the pancreatic islet as part of β-cell turnover (52). Stable overexpression of bcl-2 in rodent β-cell lines (22) and virally mediated overexpression of bcl-2 in human pancreatic islets (53) appear to confer protection against basal levels of apoptosis (i.e., normal cell turnover) and apoptosis induced by other mechanisms. However, stable overexpression of bcl-2 provides significant protection against cell death induced by reactive oxygen species but minimal protection against cytokine damage (22). Moreover, bcl-2 expression is not effective in protection of islets when transplanted into rodent models of autoimmune diabetes (54), suggesting that other, nonapoptotic mechanisms of cell damage play an important role. The current study points to nonapoptotic, cytokine-induced β-cell killing as a pathway deserving of more intensive investigation when developing strategies for protection of β-cells, either endogenous or transplanted, in the context of type 1 diabetes. Studies are underway in our laboratory with INS-1–derived cell lines that are resistant or sensitive to cytokine-mediated cytotoxicity (16) as a system for identifying genes and other factors that can provide protection against this mode of damage.

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## ACKNOWLEDGMENTS

This work was supported by National Institutes of Health Grant DK55188-04 (to C.B.N.) and an American Diabetes Association Mentor-Based Postdoctoral Fellowship Award (to J.J.C.).

We thank Dr. Jeffrey Rathwell for critical reading of the manuscript, Helena Winfield and Dr. Danhong Lu for isolation of rat islets, and Dr. Ken Walsh (Boston University) for the myrAkt1 adenovirus.


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