Islet β-Cells Deficient in Bcl-xL Develop but Are Abnormally Sensitive to Apoptotic Stimuli

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OBJECTIVE—Bcl-xL is an antiapoptotic member of the Bcl-2 family of proteins and a potent regulator of cell death. We investigated the importance of Bcl-xL for β-cells by deleting the *Bcl-x* gene specifically in β-cells and analyzing their survival in vivo and in culture.

RESEARCH DESIGN AND METHODS—Islets with β-cells lacking the *Bcl-x* gene were assessed in vivo by histology and by treatment of mice with low-dose streptozotocin (STZ). Islets were isolated by collagenase digestion and treated in culture with the apoptosis inducers staurosporine, thapsigargin, γ-irradiation, proinflammatory cytokines, or Fas ligand. Cell death was assessed by flow cytometric analysis of subgenomic DNA.

RESULTS—Bcl-xL–deficient β-cells developed but were abnormally sensitive to apoptosis induced in vivo by low-dose STZ. Although a small proportion of β-cells still expressed Bcl-xL, these did not have a survival advantage over their Bcl-xL–deficient neighbors. Islets appeared normal after collagenase isolation and whole-islet culture. They were, however, abnormally sensitive in culture to a number of different apoptotic stimuli including cytotoxic drugs, proinflammatory cytokines, and Fas ligand.

CONCLUSIONS—Bcl-xL expression in β-cells is dispensible during islet development in the mouse. Bcl-xL is, however, an important regulator of β-cell death under conditions of synchronous stress. Bcl-xL expression at physiological levels may partially protect β-cells from apoptotic stimuli, including apoptosis because of mediators implicated in type 1 diabetes and death or degeneration of transplanted islets. ***Diabetes*** **58:2316–2323, 2009**

Islet β-cells undergo apoptosis during developmental remodeling and under conditions of stress, such as islet isolation or exposure to proinflammatory cytokines or cytotoxic drugs. Members of the *Bcl-2* gene family encode proteins that function either to inhibit or promote apoptotic cell death. Of the antiapoptotic members (Bcl-xL, Bcl-2, Bcl-w, Mcl-1, and A1), readily detectable levels of Bcl-xL and Mcl-1 have been found in mouse and/or human primary β-cells by immunohistology or in situ hybridization (1,2). In contrast, Bcl-2 expression in primary β-cells appears less abundant (1,3,4), consistent with the finding that Bcl-2 and Mcl-1 are differentially expressed in epithelial structures (5). There is preliminary but unvalidated evidence for in situ expression of Bcl-w in human β-cells (Human Protein Atlas: Q92843), and in situ expression of A1 in these cells has yet to be examined. Overexpression of Bcl-xL (1,6) or Bcl-2 (7) in mouse β-cells did not have notable consequences for islet development nor did it cause neoplastic transformation of β-cells. In contrast, Mcl-1 overexpression resulted in islet hyperplasia (8). Studies of mice with global deletion of *Bcl-2* (9,10), *Bcl-w* (11), or *A1-a* (12) did not report any obvious islet abnormalities, and because mice lacking *Bcl-x* or *Mcl-1* die during embryogenesis (13,14) their roles in β-cell development and apoptosis need to be assessed in gene-targeted mice in which these genes can be deleted in a cell type–specific manner using suitable Cre transgenes.

The *Bcl-x* (*Bcl2l1*) gene encodes several isoforms (Bcl-x_L, Bcl-x_S, Bcl-x_γ, Bcl-x_β, and Bcl-$x_{\Delta TM}$), with Bcl-xL being predominant. Deletion of the *Bcl-x* gene in mice (that prevents expression of all isoforms of Bcl-x) results in embryonic lethality at around E14.5, involving massive death of neurons and immature erythroid cells (13). Cre-mediated deletion of *Bcl-x* has revealed its importance in specific cell types and developmental stages, including late stages of erythropoiesis (15), primordial germ cells (16), mammary epithelial cells during the first stage of involution (17), dendritic cells (18), immature thymocytes (19), and hepatocytes (20).

Given its importance in many cell types, we wanted to determine the role of Bcl-xL in islet β-cells during development and in culture after exposure to a variety of stress-inducing stimuli. This information would help define which apoptotic stress responses, relevant to type 1 diabetes, are controlled by Bcl-xL and whether manipulating Bcl-xL levels would prove useful for improving islet isolation, transplantation, or resistance to the toxic effects of immunosuppressive drugs. We found that islets lacking Bcl-xL appeared normal embryonically and in adults. However, Bcl-xL was needed to help protect islets from low-dose streptozotocin (STZ) treatment in vivo. In vitro assays showed that whole islets with Bcl-xL deficient β cells were stable in culture but were abnormally sensitive to a number of stressors, including cytotoxic drugs and death receptor ligation.

RESEARCH DESIGN AND METHODS

Animals were housed under specific pathogen-free conditions at the University of Melbourne and at St. Vincent's Institute (Melbourne, Australia). Experiments involving animals were conducted according to our institutional animal ethics committee guidelines. Conditional *Bcl-x* knockout mice (*Bcl-$x^{fl/fl}$*) (B6;129S6-Bcl2l1$^{tm1.1Mam}$), a gift from Dr. Lothar Hennighausen (National Institutes of Health) (16), were backcrossed to C57BL/6 (BL/6) for six generations and then bred to an N10 B6.RIP-Cre transgenic line (B6.Cg-Tg(Ins2-cre)25Mgn/J) (21) to generate mice that lacked Bcl-xL expression in

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β-cells (RIP2-Cre.*Bcl-x*$^{fl/fl}$) and littermate controls (*Bcl-x*$^{fl/fl}$). B6^{bm1}.RIP-Bcl-2 transgenic mice (B6-*H2*^{bm1}-Tg(RIP2-*Bcl*II)407Wehi/J) expressing human Bcl-2 in β-cells under control of the rat insulin promoter have been described previously (7). PCR screening of mice is detailed in the online supplemental data available at http://diabetes.diabetesjournals.org/content/early/2009/06/23/db08-1602/suppl/DC1.

Detection of *Bcl-x*$^{fl/fl}$ recombination in sorted β-cells and other tissues. Pancreatic β-cells and non–β-cells were sorted from trypsin-dissociated islets based on auto-fluorescent profile (β-cells have high FL1 auto-fluorescence) (22) using a FACSAria (Becton Dickinson, San Jose, CA). DNA was extracted from sorted islet cells and from homogenized tissues using proteinase K. *Bcl-x*$^{fl/fl}$ gene deletion was detected by PCR as detailed in the online supplemental data.

Western blotting. Isolated islets or sorted β- and non–β-cells were solubilized in lysis buffer (0.25M Tris–HCl, pH 6.8, 10% SDS, 20% glycerol, 5% 2-mercaptoethanol, 0.02% bromophenol blue, and 0.5 mg/ml Pefabloc). Wild-type mouse embryonic fibroblasts (MEFs) and MEFs from *Bcl-x* knockout mice were used as controls. After SDS-PAGE electrophoresis (12% gel) and transfer to immobilon P membrane (Billerica, MA), the blot was probed for Bcl-xL, followed by stripping and reprobing for β-actin. Antibody details are given in the online supplemental data.

Intracellular insulin staining, immunohistology, and double-label immunofluorescence. Details of the antibodies used and their dilutions are given in the online supplemental data. Intracellular insulin staining and flow cytometric analysis was performed as described (23).

Immunohistology was done on Bouin's solution fixed, paraffin-embedded pancreas. Sections (5 microns) were cut at four levels separated by 100 microns, so each section sampled different islets. For scoring of islet sizes, the numbers of insulin-expressing cells per islet were counted and the islet assigned to a size group ranging from <5 insulin-positive cells per islet to >150 insulin-positive cells per islet. Each size group was then expressed as a percentage of total islets.

Double-label immunofluorescence was done on acetone-fixed, frozen sections of pancreata. Sections were double stained for insulin and Cre recombinase. To score Cre expressing insulin cells, at least 1,000 insulin-positive cells were counted per mouse pancreas. Counting was done using a BIORAD MRC 1024 confocal microscope using BIORAD software.

TUNEL analysis of islets from STZ-treated mice. RIP-Cre.*Bcl-x*$^{fl/fl}$ mice and *Bcl-x*$^{fl/fl}$ littermate controls were injected intraperitoneally with 35 μg/ml of STZ (Sigma-Aldrich, St. Louis, MO) in citrate buffer daily for 4 days. Sections of 4% (wt/vol) paraformaldehyde fixed, paraffin-embedded pancreas were then subject to TUNEL using biotinylated dUTP that was detected with a horseradish peroxidase–streptavidin conjugate. Staining with anti-insulin antibody was detected with an alkaline phosphatase–conjugated secondary antibody. Antibody details are given in the online supplemental data. At least 45 islets (excluding islets with <20 cells) were scored, per mouse, for TUNEL-positive cells. Data were plotted as the average number of TUNEL-positive cells per islet section.

Cell death assays. Islets were isolated from 3 to 4 aged-matched male and female mice and pooled. Following overnight culture, 100 islets of uniform size range were handpicked into 3.5-cm untreated Petri dishes containing 1.1 ml of supplemented CMRL medium, and death agents were added. Islets plus medium containing detached cells were washed in PBS and dissociated at 37°C in Accutase (Chemicon, Millipore, Temecula, CA) for 5 min. After washing in PBS, islet cells were resuspended in 300 μl of a hypertonic buffer containing 50 μg/ml propidium iodide, 0.1% wt/vol tri-sodium citrate, and 0.1% vol/vol Triton-X 100 before being analyzed by flow cytometry for the numbers of cells with a <2C DNA content (apoptotic cells), as previously described (24). Cells were analyzed on a FACSCalibur using Cell Quest software (Becton Dickinson, San Jose, CA) in the FL3 channel.

Apoptosis-inducing reagents used were recombinant murine interferon γ (IFNγ) (Genentech, South San Francisco, CA), recombinant human interleukin-1β (IL-1β) (R&D Systems, Minneapolis, MN), Mega Fas ligand (FasL) (APO-O10; Apoxis/Topotarget, Lausanne, Switzerland), staurosporine (Sts; Sigma-Aldrich) dissolved at 10 mmol/l in DMSO and stored under nitrogen gas at −70°C, thapsigargin (Thap; Calbiochem, EMD Biosciences, San Diego, CA) dissolved at 1.5 mmol/l in 100% ethanol and stored at −20°C and N^{G}-methyl-L-arginine acetate salt (NMMA) (Sigma-Aldrich) dissolved at 200 mmol/l in medium and stored at −20°C.

Quantitative RT-PCR analysis of expression of antiapoptotic Bcl-2 family members. Islets from *Bcl-x*$^{fl/fl}$ and RIP-Cre.*Bcl-x*$^{fl/fl}$ mice were freshly isolated and snap frozen on dry ice. RNA was prepared using the RNeasy Kit (Qiagen, Valencia, CA). First-strand cDNA was prepared from 0.5 to 1.5 μg RNA using the Taqman RT system (Roche, Mannheim, Germany). Real-time PCR was performed using the ABI Prism 7900 (Applied Biosystems) and the QuantiTect SYBR Green PCR Kit (Qiagen) in 15-ml reaction volumes. Bcl-x, Bcl-2, Bcl-w, Mcl-1, and A1 were assayed by quantitative RT-PCR. Data analyses were performed with the ΔC_T method using β-actin as an internal control. Primer sequences are provided in online supplemental data.

Statistical analysis. Data are represented as means ± SD. Results were analyzed using a two-tailed Student's *t* test. A *P* value of <0.05 was considered significant.

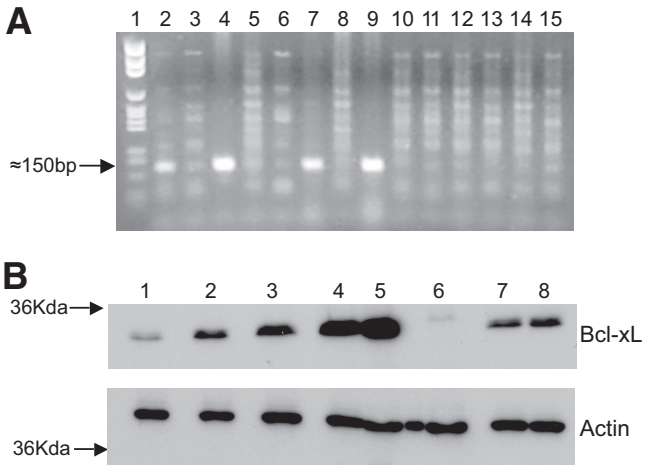

FIG. 1. *A*: PCR screening of tissues from RIP-Cre.*Bcl-x*$^{fl/fl}$ mice. Primers flanking the upstream and downstream loxP sites in the *Bcl-x* locus were used to identify the deleted *Bcl-x* allele (150-bp product) in *Bcl-x*$^{fl/fl}$ mice that expressed the Cre recombinase in β-cells (RIP promoter) or thymocytes (Lck promoter). *Lane 1*, marker; *lane 2*, RIP-Cre.*Bcl-x*$^{fl/fl}$ sorted β-cells; *lane 3*, RIP-Cre.*Bcl-x*$^{fl/fl}$ sorted non–β-cells; *lane 4*, Lck-Cre.*Bcl-x*$^{fl/fl}$ thymus; *lane 5*, Lck-Cre.*Bcl-x*$^{fl/fl}$ tail; *lane 6*, water; *lane 7*, RIP-Cre.*Bcl-x*$^{fl/fl}$ pancreas; *lane 8*, thymus; *lane 9*, salivary gland; *lane 10*, liver; *lane 11*, lung; *lane 12*, kidney; *lane 13*, heart; *lane 14*, ovary; *lane 15*, tail. Nonspecific bands in all lanes lacking a recombination product are due to oligonucleotide concatamers. *B*: Knockdown of Bcl-xL protein in β-cells from RIP-Cre.*Bcl-x*$^{fl/fl}$ mice. Sorted β-cells or whole islets from *Bcl-x*$^{fl/fl}$ and RIP-Cre.*Bcl-x*$^{fl/fl}$ mice aged 8–12 weeks were analyzed by Western blotting for Bcl-xL expression and for β-actin that served as a loading control. *Lane 1*, sorted RIP-Cre.*Bcl-x*$^{fl/fl}$ β-cells; *lane 2*, sorted *Bcl-x*$^{fl/fl}$ β-cells; *lane 3*, RIP-Cre.*Bcl-x*$^{fl/fl}$ whole islets; *lane 4*, *Bcl-x*$^{fl/fl}$ whole islets; *lane 5*, wild-type MEFs; *lane 6*, *Bcl-x* knockout MEFs; *lane 7*, sorted RIP-Cre.*Bcl-x*$^{fl/fl}$ non–β-cells; and *lane 8*, sorted *Bcl-x*$^{fl/fl}$ non–β-cells.

RESULTS

Generation of mice lacking Bcl-xL in β-cells. Mice carrying a floxed *Bcl-x* gene (16) were crossed with B6.RIP2-Cre transgenic mice (21,25), and their offspring intercrossed to obtain RIP-Cre.*Bcl-x*$^{fl/fl}$ mice and Cre-negative littermates (*Bcl-x*$^{fl/fl}$). The RIP2 promoter has been shown to direct expression of transgenes to β-cells as early as embryonic day 10 (26). *Bcl-x*$^{fl/fl}$ and RIP-Cre.*Bcl-x*$^{fl/fl}$ offspring were similar in appearance, numbers, weight, and blood glucose levels (see online supplemental data, Fig. S1*A*–*C*).

Deletion of the *Bcl-x* locus was verified by PCR on fluorescence-activated cell sorter (FACS)-sorted β-cells (~90% pure) using primers that gave rise to a ~150-bp product when the *Bcl-x* gene was deleted (Fig. 1*A*, *lane 2*). *Bcl-x* gene deletion was not seen in non–β-islet cells (Fig. 1, *lane 3*). Thymus or tail DNA from *Bcl-x*$^{fl/fl}$ mice that expressed the Cre recombinase in thymocytes under the *lck* promoter was used as a positive and negative control, respectively (Fig. 1, *lanes 4* and *5*). A survey of tissues from RIP-Cre.*Bcl-x*$^{fl/fl}$ mice showed deletion of *Bcl-x* in whole pancreas, as expected (Fig. 1, *lane 7*), and unexpectedly in salivary gland (Fig. 1, *lane 9*). The RIP-Cre transgene can show ectopic expression, for example, in the hypothalamus (25) and as our data indicate, apparently also in the salivary gland.

Western blotting of FACS-sorted β-cells (~90% pure) from RIP-Cre.*Bcl-x*$^{fl/fl}$ mice showed a substantial reduction of Bcl-xL protein when compared with β-cells from *Bcl-x*$^{fl/fl}$ mice (Fig. 1*B*, *lanes 1* and *2*). Knockdown of Bcl-xL protein was less obvious in whole islets from RIP-Cre.*Bcl-x*$^{fl/fl}$ mice when compared with *Bcl-x*$^{fl/fl}$ islets (Fig. 1, *lanes 3* and *4*) because whole islets contain a number of cell populations of which only ~75% comprise β-cells. Probing of lysates from wild-type and *Bcl-x*–deficient MEFs was used as a control for the anti–Bcl-xL antibody (Fig. 1, *lanes 5* and *6*). FACS-sorted non–β-cells showed no reduction in Bcl-xL levels in RIP-Cre.*Bcl-x*$^{fl/fl}$ islets (Fig. 1, *lanes 7* and *8*).

The RIP-Cre transgene that we used directs expression of Cre to ~85% of islet β-cells (http://jaxmice.jax.org/strain/003573.html), partially explaining the presence of some Bcl-xL protein in the RIP-Cre.*Bcl-x*$^{fl/fl}$ sorted (~90% pure) β-cell population. It was necessary to determine the efficiency of *Bcl-x* gene deletion in the β-cell population of our mice. Staining for Bcl-xL in normal islets by immunohistology or intracellular flow cytometry proved intractable. However, because Cre is a highly efficient recombinase requiring only a few molecules to mediate gene deletion (27), the presence of Cre should equate to deletion of *Bcl-x*. Pancreas sections from RIP-Cre, *Bcl-x*$^{fl/fl}$, and RIP-Cre.*Bcl-x*$^{fl/fl}$ mice were examined for Cre expression using immunofluorescence microscopy (Fig. 2*A*). *Bcl-x*$^{fl/fl}$ β-cells showed no background staining with the anti-Cre antibody. Cre expression in RIP-Cre and RIP-Cre.*Bcl-x*$^{fl/fl}$ β-cells was comparable with ~70% of adult β-cell nuclei expressing Cre at levels detectable by immunofluorescence (Fig. 2*A*, *inset*). There was variability in the intensity of Cre staining among β-cells, and the ~70% value will be an underestimate given that immunohistology would not detect low numbers of Cre molecules that may still be sufficient to mediate *Bcl-x*$^{fl/fl}$ gene deletion. Cre was also detected in neonatal and embryonic β-cells from these animals. Therefore, β-cells can develop and survive in the absence of Bcl-xL expression.

Normal histology of islets with Bcl-xL–deficient β-cells. Staining for the islet cell hormones insulin, glucagon, and somatostatin revealed normal islet architecture in RIP-Cre.*Bcl-x*$^{fl/fl}$ mice (Fig. 2*B*). We analyzed islets from recently weaned (31 days), adult (100 days), and aged mice (300 days) for the range of islet sizes. Both groups showed the same islet size range at these timepoints (Fig. 2*C*). The absolute numbers of islets counted in four histology sections were also comparable (Fig. 2*D*).

Bcl-xL–deficient β-cells are abnormally sensitive to STZ in vivo. Mice with Bcl-xL–deficient β-cells were treated with multiple low doses of STZ to induce apoptosis in the β-cells (28). TUNEL staining of pancreas sections by immunohistology showed that islets with β-cells deficient in Bcl-xL were more susceptible to this treatment than islets from control littermates (Fig. 2*E*). Thus, under conditions of stress, β-cells require Bcl-xL to maintain survival in vivo.

Effects of collagenase isolation on islets with Bcl-xL–deficient β-cells. After collagenase isolation, the islet yields from B6.RIP-Cre, *Bcl-x*$^{fl/fl}$, and RIP-Cre.*Bcl-x*$^{fl/fl}$ littermates were counted and no major differences found (Fig. 3*A*). In addition, islets from all strains appeared microscopically similar postisolation and also after 6 days in culture (online supplemental Fig. S2*A*). The proportion of β-cells in the islets was quantified by intracellular insulin staining and flow cytometric analysis. All groups contained similar proportions of insulin-producing cells (Fig. 3*B* and online supplemental Fig. S2*B*). These results show that Bcl-xL is not essential for maintaining survival of β-cells during whole-islet extraction and culture.

Effects of stress stimuli on Bcl-xL–deficient β-cells in vitro. We next tested the importance of Bcl-xL expression in cultured β-cells under conditions of stress, using whole-islet assays. We compared islets containing Bcl-xL–deficient β-cells with those containing wild-type β-cells or β-cells overexpressing Bcl-2. We expected that Bcl-2 would act as a functional homolog of Bcl-xL (as it does in hemopoietic cells) and allow us to compare β-cells that lacked Bcl-xL with those that overexpressed its functional homolog. We chose stress stimuli, relevant to type 1 diabetes, known to activate different members of the BH3-only, proapoptotic Bcl-2 protein subfamily (29). These included cytotoxic drugs (Sts) and proinflammatory cytokines (IL-1β plus IFNγ) as well as agents that induce endoplasmic reticulum stress (Thap), DNA damage (γ-irradiation [RAD]), or death receptor signaling (FasL) (29).

Loss of Bcl-xL does not accelerate Sts-induced killing of β-cells in vitro. The broad-spectrum, protein kinase inhibitor, Sts, is a classic apoptosis initiator in most cell types. Islets were cultured for 40 h in Sts, trypsinized, and then assayed for apoptosis by flow cytometric analysis of DNA fragmentation, as described by Nicoletti (24). Bcl-xL deficiency did not enhance islet cell death in response to Sts (Fig. 4*A*). We ensured that the vehicle control (DMSO) was not itself toxic to Bcl-xL–deficient β-cells (Fig. 4*B*). Overexpression of Bcl-2 protected β-cells from Sts as previously shown (7) (Fig. 4*C*). Although supra-physiological levels of Bcl-2 protected against Sts-induced killing, the presence or absence of physiological levels of Bcl-xL made no difference in the response of islet cells to this cytotoxic agent.

Bcl-xL–deficient β-cells are abnormally sensitive to treatment with the endoplasmic reticulum stress–inducing drug Thap or γ-RAD in vitro. Thap inhibits endoplasmic reticulum–specific Ca^{2+} ATPase function causing apoptosis because of endoplasmic reticulum stress. Whole islets with β-cells lacking Bcl-xL were more susceptible to Thap-induced death than control islets (Fig. 4*D*), supporting the idea that Bcl-xL is a critical player in controlling endoplasmic reticulum stress–induced apoptosis. The vehicle control (1.3% ethanol) was not toxic to Bcl-xL–deficient β-cells (Fig. 4*E*). Overexpression of Bcl-2 resulted in a reduced but not significant protection of β-cells from Thap (Fig. 4*F*). This result differs from that of Zhou et al. (6) who found that Bcl-xL overexpression in β-cells gave good protection of mouse islets from Thap-induced apoptosis after 48 h in culture. Islets were also treated with γ-RAD to induce DNA damage. Although only a low amount of islet cell death was found in control samples 6 days after RAD, this increased when Bcl-xL was lacking from β-cells (Fig. 4*G*).

In summary, Bcl-xL is needed to help protect β-cells from agents that induce endoplasmic reticulum stress or DNA damage. In addition, it appeared that in β-cells Bcl-2 and Bcl-xL may not be functionally equivalent.

Bcl-xL–deficient β-cells are abnormally sensitive to cytokine-induced apoptosis in whole-islet culture. Whole rodent primary islets undergo cell death from upregulation of inducible nitric oxide synthase (iNOS) when exposed to high levels of inflammatory cytokines, such as IL-1 plus IFNγ. NO may inhibit mitochondrial electron transfer by inactivating aconitase (see 30) result-

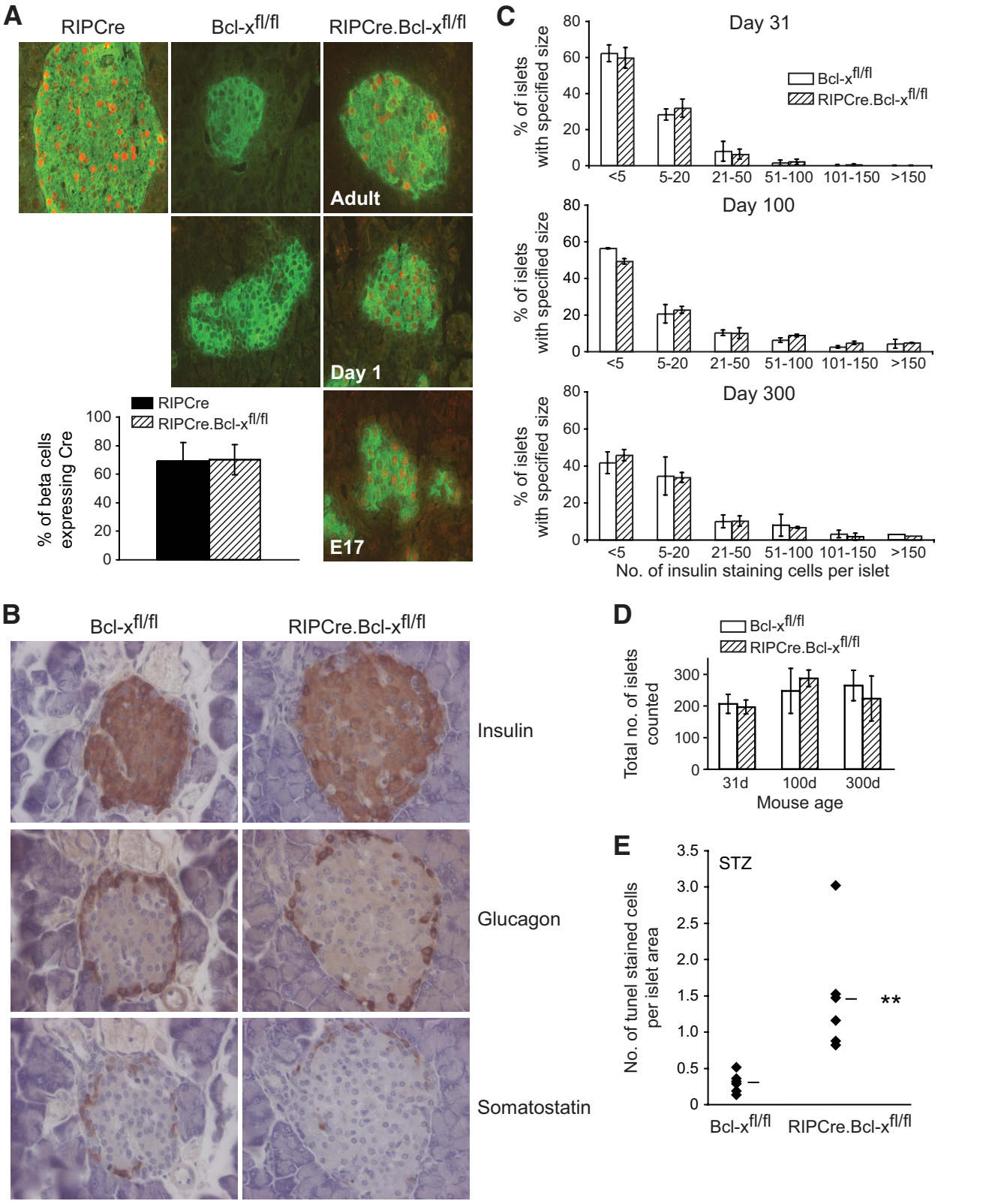

FIG. 2. Islets with Bcl-xL–deficient β-cells show normal morphology in situ but are more sensitive to low-dose STZ. *A*: Frozen pancreas sections from RIP-Cre, *Bcl-x*$^{fl/fl}$, and RIP-Cre.*Bcl-x*$^{fl/fl}$ mice were double-stained for expression of Cre (red color) and insulin (green color) (×400). The intensity of Cre staining was variable from nuclei to nuclei. *Inset*: Quantification of adult insulin-positive cells expressing Cre in RIP-Cre or RIP-Cre.*Bcl-x*$^{fl/fl}$ islets; *n* = 3 mice per group. *B*: Bouin's fixed pancreas sections stained for insulin, glucagon, and somatostatin using immunohistochemistry (brown color) (×400). *C*: Analysis of islet sizes. The numbers of insulin-positive cells per islet were counted, and the islets were categorized into sizes from <5 insulin cells per islet to >150 insulin cells per islet. Each size group was then expressed as a percentage of total islets; *n* = 3 female mice per group. *D*: Numbers of islets (including those with <5 insulin cells) found in four histology sections each separated by 100 microns. *E*: TUNEL staining by immunohistology of islets from *Bcl-x*$^{fl/fl}$ littermates and RIP-Cre.*Bcl-x*$^{fl/fl}$ mice treated with multiple low doses of STZ; *n* = 6 mice per group (three female plus three male mice). Bars represent means ± SD. ***P* < 0.01. (A high-quality digital representation of this figure is available in the online issue.)

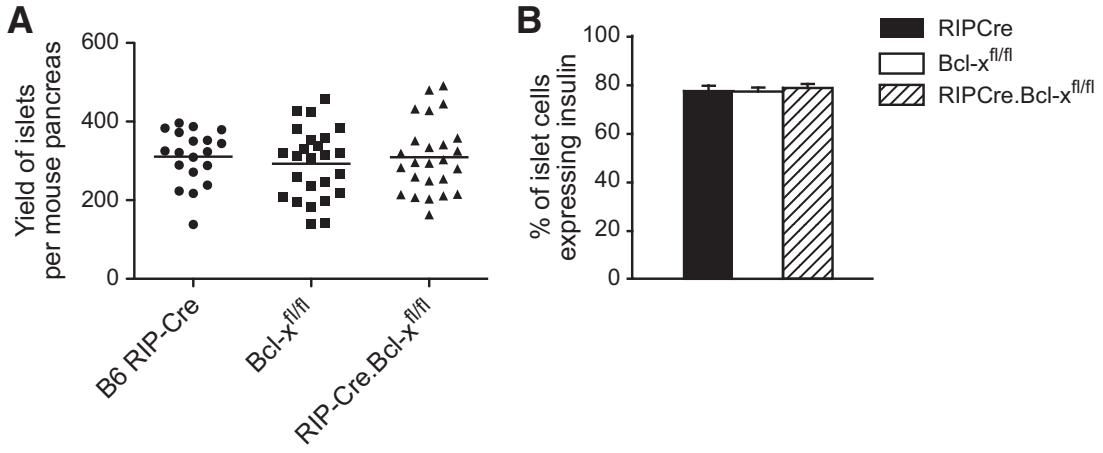

FIG. 3. Yield and intracellular insulin staining of islets with Bcl-xL–deficient β-cells. *A*: Islet yields. Islet preparations were made from 6- to 13-week-old male and female RIP-Cre, *Bcl-x$^{fl/fl}$* and RIP-Cre.*Bcl-x$^{fl/fl}$* mice. Each dot represents the islet yield from an individual mouse. *B*: Flow cytometric analysis of intracellular insulin staining. Dissociated islet cells were fixed and stained for insulin. Percentages of islet cells staining for insulin (average for three mice per group) are indicated.

ing in cell death. NO may also induce endoplasmic reticulum stress in mouse or rat β-cells (31,32) and deficiency in the endoplasmic reticulum stress–induced protein, C/EBP homologous protein, can partially protect (31). Antiapoptotic Bcl-2 family members, such as Bcl-xL or Bcl-2, are likely to be critical for protecting islets against both of these apoptotic pathways. We found that islets with β-cells lacking Bcl-xL were more susceptible to IL-1β plus IFNγ (Fig. 5*A*). This death could be blocked in both control and Bcl-xL–deficient islets by coaddition of the

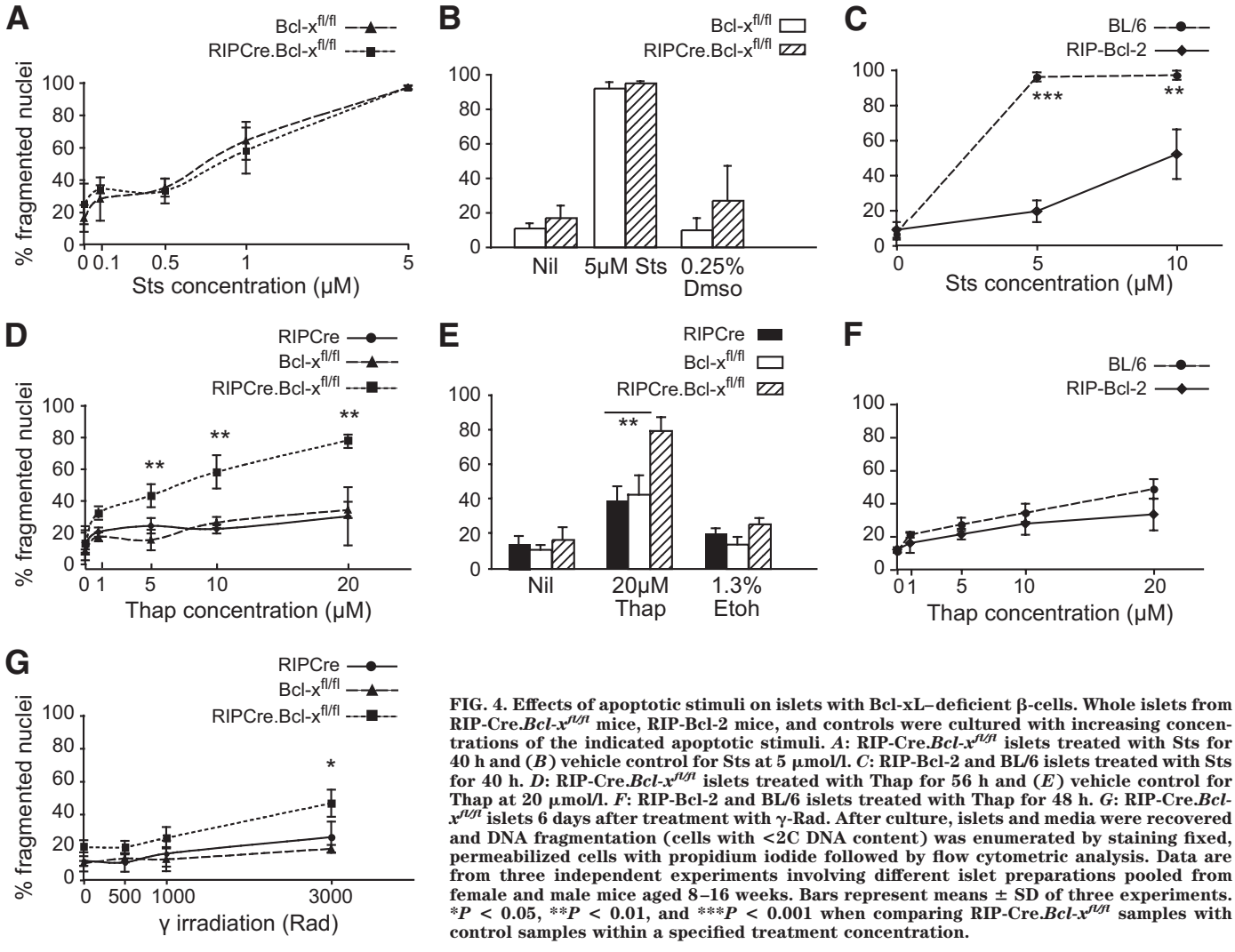

FIG. 4. Effects of apoptotic stimuli on islets with Bcl-xL–deficient β-cells. Whole islets from RIP-Cre.*Bcl-x$^{fl/fl}$* mice, RIP-Bcl-2 mice, and controls were cultured with increasing concentrations of the indicated apoptotic stimuli. *A*: RIP-Cre.*Bcl-x$^{fl/fl}$* islets treated with Sts for 40 h and (*B*) vehicle control for Sts at 5 μmol/l. *C*: RIP-Bcl-2 and BL/6 islets treated with Sts for 40 h. *D*: RIP-Cre.*Bcl-x$^{fl/fl}$* islets treated with Thap for 56 h and (*E*) vehicle control for Thap at 20 μmol/l. *F*: RIP-Bcl-2 and BL/6 islets treated with Thap for 48 h. *G*: RIP-Cre.*Bcl-x$^{fl/fl}$* islets 6 days after treatment with γ-Rad. After culture, islets and media were recovered and DNA fragmentation (cells with <2C DNA content) was enumerated by staining fixed, permeabilized cells with propidium iodide followed by flow cytometric analysis. Data are from three independent experiments involving different islet preparations pooled from female and male mice aged 8–16 weeks. Bars represent means ± SD of three experiments. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ when comparing RIP-Cre.*Bcl-x$^{fl/fl}$* samples with control samples within a specified treatment concentration.

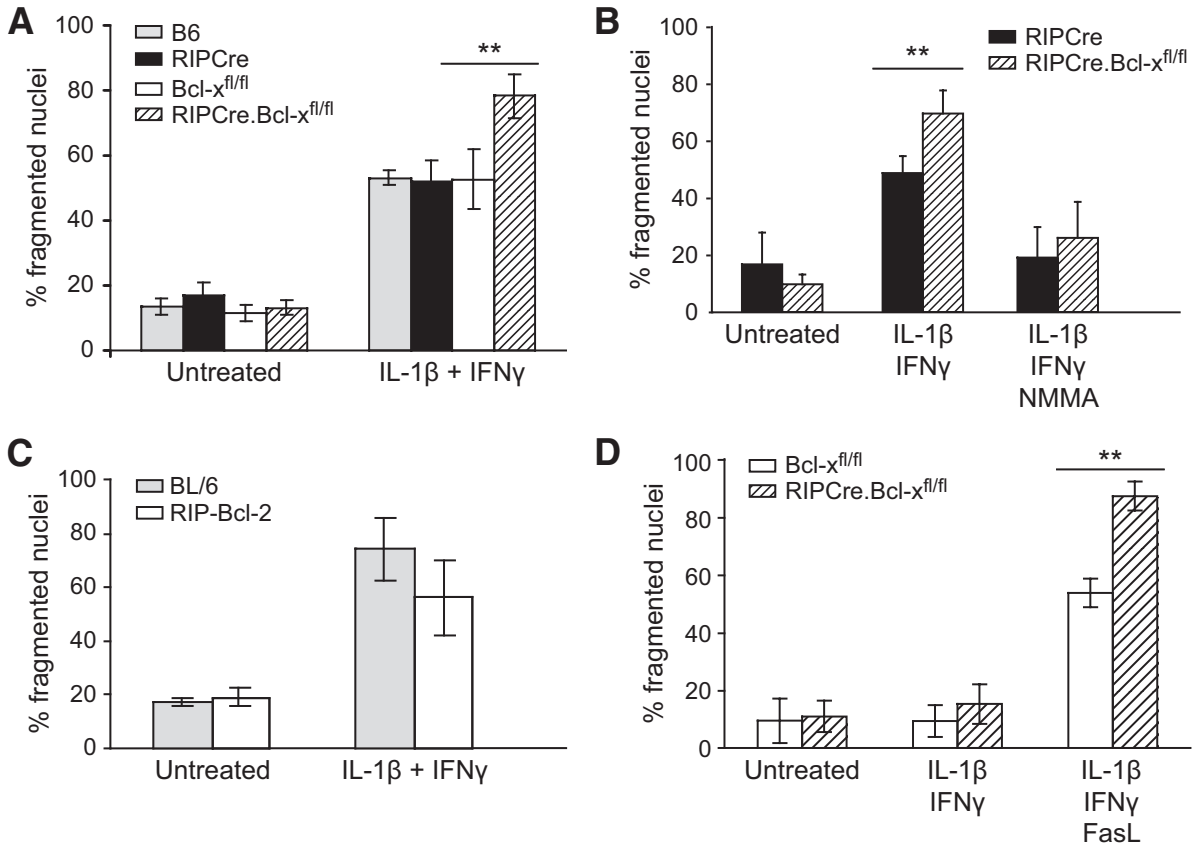

FIG. 5. Effects of cytokines or FasL on islets with Bcl-xL–deficient β-cells. Whole islets from *Bcl-x*$^{fl/fl}$ and RIP-Cre.*Bcl-x*$^{fl/fl}$ mice were cultured with high doses of cytokines or alternatively with FasL. *A*: IL-1β (100 units/ml) plus IFNγ (100 units/ml) for 3–4 days. *B*: IL-1β plus IFNγ for 3–4 days in the presence of NMMA (2 mmol/l). *C*: RIP-Bcl-2 and BL/6 islets cultured with IL-1β (100 units/ml) plus IFNγ (100 units/ml) for 3–4 days. *D*: Mega FasL (100 nmol/l) plus IL-1β (150 units/ml) and IFNγ (2 units/ml) for 4 days. After treatment, islets and media were recovered and cell survival was measured as described in Fig. 4. Data are from three individual experiments involving different islet preparations pooled from female and male mice aged 8–16 weeks. Bars represent means ± SD of three independent experiments. **$P < 0.01$ when comparing RIP-Cre.*Bcl-x*$^{fl/fl}$ samples with control samples within a specified treatment.

iNOS inhibitor NMMA (Fig. 5*B*), indicating that the increased β-cell death in Bcl-xL–deficient islets was mediated primarily by NO. Overexpression of Bcl-2 in β-cells did not protect the cytokine-treated islets from death as previously shown (see 33 and Fig. 5*C*).

Bcl-xL–deficient β-cells are abnormally sensitive to FasL-induced apoptosis in vitro. Islet cells do not normally express Fas, but low concentrations of IL-1 plus IFNγ (that do not kill) can induce Fas on β-cells (34). After culture with low levels of IL-1β plus IFNγ to induce Fas and with FasL to induce cell killing, death was substantially increased in islets with β-cells that lacked Bcl-xL (Fig. 5*D*). Our lab has previously shown that β-cell death through the death receptor pathway is mediated by the BH3-only protein Bid and can be inhibited by Bcl-2 overexpression (35). In summary, Bcl-xL is critical for protection of β-cells from death through the death receptor pathway.

Quantitative RT-PCR analysis of Bcl-xL–deficient β-cells did not reveal upregulation of other Bcl-2 family antiapoptotic members. Given there was so little impact of Bcl-xL deficiency on the development of islets and their survival in the absence of cytotoxic stressors, we tested whether other antiapoptotic members of the Bcl-2 family were transcriptionally upregulated in the absence of Bcl-xL. Whole islets from *Bcl-x*$^{fl/fl}$ and RIP-Cre.*Bcl-x*$^{fl/fl}$ mice were assayed for levels of mRNA for antiapoptotic Bcl-2 family members (Bcl-xL, Bcl-2, Bcl-w, Mcl-1, and A1). As expected, there was a notable reduction in Bcl-xL mRNA in islets with Bcl-xL–deficient β-cells (Fig. 6) consistent with the Western analysis of whole islets (Fig. 1*B*). Unexpectedly, a twofold reduction in Bcl-2 mRNA

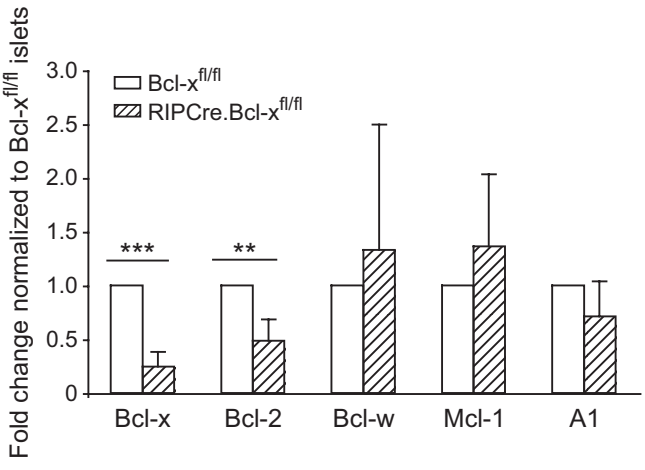

FIG. 6. Differences in the levels of RNA expression of Bcl-2 family antiapoptotic members in islets with Bcl-xL–deficient β-cells. Whole islets from *Bcl-x*$^{fl/fl}$ and RIP-Cre.*Bcl-x*$^{fl/fl}$ mice were assayed for Bcl-xL, Bcl-2, Bcl-w, Mcl-1, and A1 mRNA levels. Relative RNA expression levels were calculated by normalizing to the signal for β-actin in each sample and then dividing the RIP-Cre.*Bcl-x*$^{fl/fl}$ by the *Bcl-x*$^{fl/fl}$ value. Means ± SD of four independent experiments involving different islet preparations pooled from female and male mice aged 8–10 weeks. Reduction in the level of Bcl-xL mRNA was observed, ***$P < 0.00003$ and Bcl-2 **$P < 0.002$.

was also noted, but the biological implications of this reduction are at present unclear. There was no significant upregulation of the other gene transcripts in islets with Bcl-xL–deficient β-cells.

DISCUSSION

To address the role of Bcl-xL in pancreatic islets we used the Cre-loxP system to delete the *Bcl-x* gene specifically in β-cells. Loss of Bcl-xL in β-cells was not lethal. The numbers of islets were normal and the proportion of β-cells that made up the islets was similar to controls. Although a proportion of β-cells (~20%) do not express Cre (25) and would remain *Bcl-x* sufficient, these cells did not have a survival advantage over their *Bcl-x*–deficient neighbors, again indicating that Bcl-xL is not essential for β-cell growth and survival. Although other prosurvival members were expressed but not transcriptionally upregulated in response to Bcl-xL loss in β-cells, it appears likely that normal levels could substitute for Bcl-xL deficiency, with Mcl-1 being a plausible candidate given its ready detection in islets at the protein level (2,5). Interestingly, Hager et al. (36) recently showed that *Bcl-x* gene deficiency did not affect growth of β-cell tumors transformed by the SV40 T antigen and that other antiapoptotic members were not upregulated at the transcriptional level in these tumors.

Our findings in β-cells differ from those for hematopoietic cells (13,15), neurons (13,37,38), and germ cells (16) in which Bcl-xL was found to play an indispensable role in cell survival during development. Our results are more like those observed for another epithelial tissue, the mammary gland (17), where Bcl-xL depletion did not affect normal organ development or function during mammopoiesis (17), although it appears to be the most highly expressed antiapoptotic Bcl-2 family member in this tissue (17,39). Bcl-xL was important, however, during the first phase of mammary gland involution after weaning, a stage characterized by considerable cell stress and extensive tissue remodeling that is associated with apoptosis (17,40). In line with this, Bcl-xL–deficient β-cells were abnormally susceptible to death when subject to a synchronous cell stressor, low-dose STZ.

In vitro, Bcl-xL helped to protect β-cells against a variety of cell death inducers that activate different proapoptotic, BH3-only proteins (29). Cell death elicited by Thap-induced endoplasmic reticulum stress, γ-RAD, IL-1β plus IFNγ, or Fas death receptor signaling was enhanced in the absence of Bcl-xL, confirming it as a critical guardian of cell survival in these settings. Unexpectedly, Bcl-xL deficiency did not enhance cell death by the classic apoptosis initiator, Sts. Sts may cause a massive induction of several BH3-only proteins that rapidly overwhelms all antiapoptotic Bcl-2 family members in β-cells so that presence or absence of physiological levels of Bcl-xL makes little difference.

We also studied the effects of Bcl-2 overexpression in β-cells under the premise that Bcl-2 was a functional homologue of Bcl-xL. Bcl-2 overexpression reduced cell deaths induced by Sts and Fas ligand (see 35) but not by Thap or cytokines (IL-1β plus IFNγ). It is possible that in response to Thap or cytokines β-cells activate additional apoptotic death pathways not controlled by Bcl-2 family members, for example serine protease-mediated mechanisms that damage the mitochondrial membrane (41). Alternatively, Zhou et al. (6) found that overexpression of Bcl-xL itself did afford β-cells significant protection from Thap-induced killing. It may be that Bcl-2 and Bcl-xL can play different roles in the β-cell's response to different types of cytotoxic insults. An explanation for this comes from studies on mouse embryonic fibroblasts (42) which reported that although Bcl-2 and Bcl-xL can act as functional homologues under some situations, they in fact control the Bax/Bak molecules differently. Bcl-xL is able to restrain both Bak and Bax, whereas Bcl-2 can only restrain Bax and may therefore be a less effective inhibitor of apoptosis under certain conditions.

In conclusion, we have demonstrated that although Bcl-xL is not needed for β-cells to develop, it plays an important role in promoting islet cell survival when islets are exposed to a range of death stimuli. Our data indicate that Bcl-xL is critical for helping to protect β-cells against most, but not all, specific death signals. In addition, Bcl-xL and Bcl-2 are not functionally equivalent in β-cells. Whether Bcl-xL also functions to maintain islet integrity under unfavorable culture conditions will be important to examine for the prospects of clinical applications of islet manipulation in culture followed by subsequent transplantation into patients.

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