Bcl-2 and Bcl-xL Suppress Glucose Signaling in Pancreatic β-Cells

Dan S. Luciani,1,2,3 Sarah A. White,1,2,3 Scott B. Widenmaier,1 Varun V. Saran,4,5 Farnaz Taghizadeh,1 Xiaoke Hu,1 Michael F. Allard,4,5 and James D. Johnson1,2

B-cell lymphoma 2 (Bcl-2) family proteins are established regulators of cell survival, but their involvement in the normal function of primary cells has only recently begun to receive attention. In this study, we demonstrate that chemical and genetic loss-of-function of antiapoptotic Bcl-2 and Bcl-xL significantly augments glucose-dependent metabolic and Ca2+ signals in primary pancreatic β-cells. Antagonism of Bcl-2/Bcl-xL by two distinct small-molecule compounds rapidly hyperpolarized β-cell mitochondria, increased cytosolic Ca2+, and stimulated insulin release via the ATP-dependent pathway in β-cell under substimulatory glucose conditions. Experiments with single and double Bax–Bak knockout β-cells established that this occurred independently of these proapoptotic binding partners. Pancreatic β-cells from Bcl-2−/− mice responded to glucose with significantly increased NAD(P)H levels and cytosolic Ca2+ signals, as well as significantly augmented insulin secretion. Inhelicde deletion of Bcl-xL in adult mouse β-cells also increased glucose-stimulated NAD(P)H and Ca2+ responses and resulted in an improvement of in vivo glucose tolerance in the conditional Bcl-xL knockout animals. Our work suggests that prosurvival Bcl proteins normally dampen the β-cell response to glucose and thus reveals these core apoptosis proteins as integrators of cell death and physiology in pancreatic β-cells.

Type 2 diabetes involves combined defects in β-cell function and mass. Therapeutic efforts to combat diabetes could benefit from a better understanding of proteins that control both β-cell physiology and apoptosis. Cell survival is tightly regulated by signaling pathways that converge on pro- and anti-apoptotic proteins from the B-cell lymphoma 2 (Bcl-2) family. Prominent antiapoptotic members include Bcl-2, Bcl-xL, and Mcl-1, whereas key apoptosis-inducing members include Bax, Bak, and the structurally distinct members Bad, Bid, and Bim (1,2). Elegant work from Danial and colleagues (3) demonstrated that Bad has a physiological role in β-cells, aside from its role in β-cell apoptosis. Specifically, Bad phosphorylated at serine 155 promotes glucose-stimulated insulin secretion via interaction with glucokinase (3). It is not known whether any other Bcl family members play roles in β-cell physiology. The best understood of the prosurvival proteins, Bcl-2 and Bcl-xL, have been implicated in the control of β-cell survival under stress (4,5), but little is known about the roles of these proteins in healthy β-cells. Studies in other cell types have suggested that Bcl-2 and Bcl-xL regulate Ca2+ homeostasis (6) and mitochondrial physiology (7,8). Given the unique roles for mitochondria and Ca2+ in β-cell glucose signaling, we hypothesized that prosurvival Bcl proteins may be important in normal β-cell function.

To date, most studies of antiapoptotic Bcl proteins in β-cells have relied on forced overexpression (9–11). In this study, we examined the roles of endogenous prosurvival Bcl proteins in pancreatic β-cells using complementary loss-of-function approaches, small molecule Bcl-2/Bcl-xL antagonists, as well as Bcl-2 and Bcl-xL knockout mice. Our results demonstrate the combined importance of Bcl-2 and Bcl-xL for mitochondrial integrity and β-cell survival and reveal distinct roles for the endogenous proteins in metabolic signaling, Ca2+ homeostasis, and insulin secretion. Our findings place these important members of the core apoptotic machinery at the interface of β-cell survival and physiology.

RESEARCH DESIGN AND METHODS

Reagents. Compound-6 (also known as Bcl-2 inhibitor) and YC137 (also known as Bcl-2 inhibitor II) from Calbiochem (La Jolla, CA) were prepared in dimethyl sulfoxide. Pura-2-AM, Rhodamine123, and MitoTracker from Life Technologies/Invitrogen (Burlington, Ontario, Canada). Propidium iodide (PI), nifedipine, diazoxide, sodium azide, and tetramethylrhodamine ethyl ester perchorlite (TMRE) were from Sigma-Aldrich (St. Louis, MO).

Imaging and flow cytometry. Dispersed islet cells and intact islets were imaged following 24–48 h culture on glass coverslips. Changes in cytosolic Ca2+, mitochondrial membrane potential (Δϕm), and NAD(P)H autofluorescence were imaged as described (12,13). Mitochondrial Ca2+, endoplasmic reticulum (ER) Ca2+, and the activation of caspase-3 were monitored in single MIN6 cells transfected with fluorescence resonance energy transfer (FRET)-based biosensors. MIN6 cell Δϕm was estimated using TMRE and flow cytometry (14). Late-stage cell death was imaged using PI (14).

Metabolism assays. To examine changes in the ATP/ADP ratio, MIN6 cells in 96-well plates were equilibrated for 30 min in Krebs Binger Buffer (KRB) containing (in mM unless otherwise noted): 119 NaCl, 4.7 KCl, 25 NaHCO3, 2.5 CaCl2, 1.2 MgSO4, 1.2 KH2PO4, 5 mg/mL radioliodonoasay-grade bovine albumin, and 3 mmol/L glucose, followed by treatment for 30 min as indicated. ATP/ADP ratios were measured using the ApoSSENSOR kit (BioVision, Mountain View, CA) using a TECAN M1000 luminometer (Tecan Group Ltd.).

Glucose oxidation rates were determined in cultures of dispersed islet cells by quantifying 14CO2 generated from metabolized 14C-labeled glucose as described (15,16). Adherent islet cells in T-25 flasks were preincubated 1 h in 3 mmol/L glucose KRB. This was followed by 1 h incubation with KRB containing 1 μCi/mL [U-14C]glucose and test concentrations of nonlabeled glucose during which 14CO2 was collected in a hyamine trap. Injection of 9 N H2SO4 stopped the reaction and released 14CO2 captured in the media as [14C]bicarbonate. Flasks were gently agitated for 2 h at room temperature before measuring captured radioactivity using a Beckman LS6500 Liquid Scintillation Counter (Beckman Coulter).

Glucose oxidation rates were normalized to total protein quantified from identical aliquots of similarly treated cells.

Mouse models. Bax−/− (Jax stock number 002894) and Bak−/− (Jax stock number 004183) mice and age- and background-matched wild-type controls were from The Jackson Laboratory (Bar Harbor, ME). Littermate Bcl-2−/−...
Bcl-2−/− and Bcl-xL−/− mice were obtained by breeding mice heterozygous for the Bcl2tm1Sjk mutation (Jax stock number 002265; The Jackson Laboratory). To generate Bcl-xL−/−;Bcl-1Cre-ERT2 mice and Bcl-xLtm1Sjk control littermates, we mated Ptx1-CreERT2 mice (17) with Bcl-xL−/− mice (exons 1 and 2 flanked by Lox P sites) (18). To create mice with double Bax and Bak deletion in their β-cells, we bred Ptx1-CreER animals with Bak−/−;Bax−/− control mice (Baxtm2Sjk; Jax stock number 006329; The Jackson Laboratory). To generate Bcl-x−/− cells, we bred Pdx1-CreER mice (17) with Bcl-x−/− (Baxtm2SjkBak1tm1Thsn/J; Jax stock number 002265; The Jackson Laboratory) and obtained Bak−/−;Bax−/− mice that resembled Bcl-x−/− mice in vivo. Animals were housed in the University of British Columbia Animal Care Committee.

Islet isolation, cell culture, and insulin secretion. Mouse islet isolation by collagenase digestion/filtration and culture have been described (19). Human islets were obtained from Dr. Garth Warnock (Vancouver General Hospital) after consent and culture as described (12). MIN6 cells were cultured in Dulbecco’s modiﬁed Eagle’s medium (25 mmol/L glucose). Insulin release was measured from dispersed mouse islet cells in 48-well plates (20). Cells were equilibrated for 60 min in 3 mmol/L glucose KRB and then stimulated for 45 min with treatments as indicated (20). Insulin secretion from batches of size-matched islets was examined by perifusion and radioimmunoassay as previously described (12).

Immunoblotting and coimmunoprecipitation. For immunoprecipitation, 500 μg of total protein was incubated overnight (4°C) with anti-Bad (catalog number 9292; Cell Signaling Technology) 1:200, followed by 3-h incubation (4°C) with protein A-agarose (Santa Cruz Biotechnology). Complexes were washed with PBS and protease inhibitors in Ultrafree-MC Filters (Millipore) and eluted using 4X SDS-PAGE in PBS with protease inhibitors. In some studies, nuclear (PARIS; Ambion Inc.) and mitochondrial fractions (Mitochondrial/Cytosol Kit; Biovision, Inc.) were obtained. Proteins were resolved by SDS-PAGE, transferred to polyvinylidene difluoride membranes, and probed overnight (4°C) for Bcl-xL, Bcl-2, and Bax antibody (catalog numbers 2762, 2870, 2772, and 4272; Cell Signaling Technology); phospho-Bad Ser112, Ser136, and Ser155 (catalog number 9291; Cell Signaling Technology); Bak; Bax; Bad (catalog numbers Baxtm2Sjk, Bcl-xL, Bcl-2, c (catalog numbers 2762, 2870, 2772, and 4272; Cell Signaling Technology); phospho-Bad Ser112, Ser136, and Ser155 (catalog numbers 9291, 4366, and 9297; Cell Signaling Technology); Bak; Bax; Bad (catalog numbers Baxtm2Sjk, Bcl-xL, Bcl-2, and Cre recombinase (Novagen).

Statistical analysis. Data are shown as mean ± SEM. Differences between two groups were compared by unpaired Student t test and multiple groups by one-way ANOVA followed by Bonferroni multiple comparison test. Differences were considered signiﬁcant if P < 0.05.

RESULTS

Kinetic characterization of apoptotic events during prolonged Bcl-2/Bcl-xL antagonism. We used small-molecule antagonists to probe moment-to-moment functions of Bcl-2 and Bcl-xL in pancreatic β-cells. The structurally distinct inhibitors, compound 6 (C6) and YC137, were originally identiﬁed by their ability to bind both Bcl-2 and Bcl-xL and displace proapoptotic members such as Bak and Bid (21,22). We ﬁrst established that BH3-displacement could also be observed in intact β-cells. Indeed, C6 caused a rapid reduction in the amount of Bcl-xL that was bound to the BH3-only protein Bad without affecting the total levels of Bad or Bcl-xL protein within 1 h (Fig. 1A and B). This conﬁrmed the expected mechanism of this antagonist on endogenous Bcl proteins in intact β-cells.

Displacement of BH3 domain proteins from Bcl-2 and Bcl-xL by YC137 or C6 sensitizes tumor cells to apoptosis, an effect that is more potent with increasing Bcl-2 expression (21,22). Unlike many tumor cells, primary β-cells do not hyperexpress Bcl-2 or Bcl-xL. We therefore tested if these inhibitors affect β-cell survival. Prolonged Bcl antagonism induced dose- and time-dependent cell death in human and mouse islet cells, as well as MIN6 β-cells (Fig. 1C–E). This involved mitochondrial apoptosis, as evidenced by redistribution of Bax from cytosol to mitochondria and release of mitochondrial cytochrome c (Fig. 1F). PI incorporation was preceded by the activation of caspase-3, imaged in real time (Fig. 1G). Of note, the ΔΨm of β-cells treated with C6 or YC137 underwent an initial hyperpolarization that suggested mitochondrial activation within the first half hour, well prior to any evidence of apoptosis. This was followed hours later by collapse of ΔΨm, demonstrating a late-stage loss of mitochondrial integrity (Fig. 1H). These results demonstrate that even in the absence of other stresses, combined and sustained antagonism of Bcl-2 and Bcl-xL initiates mitochondrial apoptosis in β-cells. Importantly, apoptosis was not detected earlier than 2 h, indicating that cellular responses occurring less than an hour after Bcl-2/Bcl-xL antagonism are separate from the central apoptotic events.

Antagonizing Bcl-2/Bcl-xL initiates KATP channel- and depolarization-dependent Ca2+ entry and insulin secretion. Remarkably, Bcl-2/Bcl-xL antagonists rapidly triggered marked Ca2+ ﬂuctuations in mouse and human islet cells that resembled Ca2+ responses to glucose (Fig. 2A–C). Similar effects were observed in MIN6 β-cells (Fig. 2D). The percentage of cells activated within 30 min of Bcl inhibition was concentration-dependent (Fig. 2E). The Ca2+ signals ceased upon washout of the inhibitor, strongly suggesting a physiological basis rather than cell damage. C6 also increased average cytosolic Ca2+ in intact islets, although the rapid ﬂuctuations were dampened (Fig. 2F). Together, these ﬁndings provide the ﬁrst direct evidence that prosurvival Bcl proteins regulate moment-to-moment calcium homeostasis in β-cells.

Next, we sought to determine the cellular site where Bcl-2 and/or Bcl-xL control Ca2+ homeostasis. In other cell types, Bcl-2 and Bcl-xL reside on the membranes of Ca2+ handling organelles, the mitochondria, ER, and the nuclear envelope (23,24). The subcellular location of Bcl-2 and Bcl-xL in β-cells has not been reported. We found that Bcl-2:GFP displayed some colocalization with mitochondria, but also had a clear nonmitochondrial distribution, likely reﬂecting ER (Fig. 3A and D). In contrast, Bcl-xL:yellow ﬂuorescent protein (YFP) showed near-exclusive colocalization with mitochondria, which at high magniﬁcation could often be seen in an apparent association with the mitochondrial membrane (Fig. 3B and D) and minimal association with ER (Fig. 3C and D). A differential distribution of endogenous Bcl-2 and Bcl-xL between mitochondrial and nonmitochondrial compartments was also found by subcellular fractionation (Fig. 3E). These ﬁndings suggest that β-cell Bcl-2 and Bcl-xL may have overlapping, but distinct, roles.

Bcl-2 and Bcl-xL influence ER Ca2+ release in other cell types (6,25,26). Imaging ER luminal Ca2+ directly (14,27), and obtained Bak−/−;Bax−/− control littermates. Ablation of Bax and Bak was achieved by injecting Ptx1-CreER-positive animals and littermate controls with 3 mg/g of tamoxifen (Sigma-Aldrich) on 5 consecutive days. Glucose tolerance and in vivo insulin secretion were assessed following intraperitoneal injection with glucose as indicated. Insulin tolerance was assessed after intraperitoneal injection of 0.75 units/kg insulin. Animals were fasted for 6 h prior to study. Studies were approved by the University of British Columbia Animal Care Committee.
FIG. 1. Small-molecule inhibition of Bcl-2/Bcl-xL rapidly displaces Bad and eventually induces mitochondrial apoptosis. A: Top: Western blot illustrating the loss of Bcl-xL coimmunoprecipitation with Bad in MIN6 β-cells treated with C6. Bottom: Densitometric quantification of the ratio of Bcl-xL to Bad protein in Bad immunoprecipitates after various durations of C6 exposure. Data (mean ± SEM) are normalized to control (n = 3–5; *P < 0.05 vs. time 0). B: Bcl-xL and Bad protein levels in MIN6 β-cells treated with 80 μmol/L C6 (n = 3; *P < 0.05 vs. time 0). C and D: PI incorporation in mouse islet cells and MIN6 β-cells during incubation with C6 (n = 3). E: Relative cell death (PI+ cells) in human islet cells treated with Bcl-2/Bcl-xL antagonists (n = 3 donor preparations). F: Western blots for Bax and cytochrome c (Cyto c) in mitochondrial and cytosolic fractions from MIN6 β-cells treated with 40 μmol/L C6 for 4 h (n = 3). G: Top: Caspase-3 activation (loss of MiCy-mKO FRET) imaged in four individual MIN6 β-cells during continued Bcl-2/Bcl-xL inhibition. Bottom: C6 (20 μmol/L) activated caspase-3 at an average time of 2.9 ± 0.3 h (n = 12 cells from two independent cultures). Staurosporine (STS; 10 μmol/L) activated caspase-3 after 1.65 ± 0.12 h (n = 10 cells from two independent cultures). H: Flow cytometric detection of mitochondrial membrane potential in MIN6 β-cells treated with 20 μmol/L C6, 20 μmol/L YC137, and 30 mmol/L glucose (30G). Reduction of TMRE intensity indicates a loss of ΔΨm (n = 3 cultures). (A high-quality color representation of this figure is available in the online issue.)
substimulatory glucose (3 mmol/L) induced a modest, but significant, elevation in insulin release similar in magnitude to that triggered by 100 \( \mu \text{mol/L} \) of the K\(_{\text{ATP}}\) channel blocker tolbutamide (Fig. 4E). These insulin-secretion responses were attenuated by diazoxide and were not additive with the response to tolbutamide (Fig. 4E). These findings indicate that blocking Bcl-2/Bcl-x\(_{L}\) increases basal mitochondrial glucose metabolism rather than acting independently of the sugar.

**Bcl-2/Bcl-x\(_{L}\) inhibition increases mitochondrial activity.**

Our results to this point indicated that the events induced by Bcl protein inhibition are similar to those that underlie \( \beta\)-cell glucose signaling. Indeed, sodium azide blocked C6-stimulated \( \text{Ca}^{2+}\) signals, establishing a requirement for mitochondrial respiratory flux (Fig. 4E). Importantly, \( \text{Ca}^{2+}\) signaling upon Bcl antagonism was suppressed in the absence of glucose (Fig. 2E), suggesting that a minimum of metabolic substrate is required to support the \( \text{Ca}^{2+}\) responses. These findings indicate that blocking Bcl-2/Bcl-x\(_{L}\) increases basal mitochondrial glucose metabolism rather than acting independently of the sugar.

To further establish if Bcl antagonists promote mitochondrial activity, we directly imaged \( \Delta \Psi \text{in} \) and mitochondrial \( \text{Ca}^{2+}\)
In agreement with our flow cytometry results, Bcl antagonism hyperpolarized the mitochondria of single primary mouse β-cells within minutes in a concentration-dependent manner (Fig. 4). The presence of Bcl-2/Bcl-xL antagonist did not prevent additional glucose-induced hyperpolarization (Fig. 4). Bcl-2/Bcl-xL inhibition also evoked reversible mitochondrial Ca²⁺ signals that resembled glucose stimulation (Fig. 4), providing further evidence for mitochondrial activation.

FIG. 3. Differential subcellular distribution of Bcl-2 and Bcl-xL in β-cells. A and B: Representative images of MIN6 cells expressing GFP-tagged Bcl-2 and YFP-tagged Bcl-xL and loaded with 100 nmol/L MitoTracker Red. C: MIN6 cell coexpressing Bcl-xL:YFP and ER-targeted monomeric red fluorescent protein (mRFP). D: Pearson correlation coefficient (coeff.) quantifying colocalization of Bcl-xL:YFP with mitochondrial dsRed (n = 6) or ER mRFP (n = 5) and Bcl-2:GFP with ER mRFP (n = 5) in MIN6 β-cells ( * P < 0.05, ** P < 0.001). E: Western blots for endogenous Bcl-2 and Bcl-xL in fractions of MIN6 β-cells (n = 3). Cytochrome c oxidase (Cox-IV) indicates the mitochondrial fraction. (A high-quality digital representation of this figure is available in the online issue.)
FIG. 4. Bcl-2/Bcl-xL antagonism stimulates β-cell mitochondrial metabolism, K<sub>ATP</sub>-dependent Ca<sup>2+</sup> entry, and insulin secretion. A: Representative recording of ER Ca<sup>2+</sup> changes in MIN6 β-cells exposed to C6 and carbachol (Cch) (n = 6 cells). Inset: MIN6 cell expressing the ER-targeted D1ER Ca<sup>2+</sup> sensor. B: Lack of C6-induced Ca<sup>2+</sup> influx in the absence of extracellular Ca<sup>2+</sup>. The basally active cell illustrates the rapid loss of Ca<sup>2+</sup> entry.
Of note, Bcl antagonism caused an increase in the cellular ratio of ATP to ADP similar to that evoked by 20 mM glucose (Fig. 4K). Our results demonstrate that acute inhibition of Bcl-2/Bcl-xL promotes mitochondrial activity in β-cells, resulting in the same events activated by stimulatory glucose. These findings implicate Bcl-2 and/or Bcl-xL in the regulation of β-cell mitochondrial physiology and suggest that one of their day-to-day roles is to suppress basal glucose metabolism.

**Genetic ablation of Bcl-2 enhances β-cell glucose responses.** To validate our experiments with small molecule inhibitors, we examined the importance of Bcl-2 and Bcl-xL in β-cell function in vitro using a genetic loss-of-function approach. First, we used islets from Bcl-2/−/− mice. These global knockout mice are approximately half the size of their wild-type or heterozygous littermates, insulin-hypersensitive, develop polyolcytic kidney disease, and die at various ages between 2 and 19 weeks (30) (D.S.L. and J.D.J., unpublished observations), precluding interpretable in vivo analysis of β-cell function. Nevertheless, we were able to isolate islets for in vitro analysis from a limited number of Bcl-2/−/− mice prior to any signs of illness and compare them to islets from phenotypically normal heterozygous and wild-type littermates (31). Real-time PCR confirmed the loss of Bcl-2 expression in Bcl-2/−/− and Bcl-2+/− islets relative to Bcl-2+/+ controls, with no compensatory increase in Bcl-xL (Fig. 5A). Bcl-2/−/− and Bcl-2+/− β-cells showed enhanced sensitivity to a stepwise glucose ramp stimulus relative to cells from wild-type littermates (Fig. 5B and C). Intact islets from Bcl-2/−/− mice also showed increased Ca2+ and metabolic NAD(P)H responses to 6 mM glucose (Fig. 5E and F). In perfusion experiments, we observed significantly increased insulin secretion from Bcl-2/−/− islets in response to 10 and 15 mM glucose, compared with Bcl-2+/− islets (Fig. 5G). Loss of Bcl-2 had no effect on the responses to depolarization with KCl, further indicating a change at the level of β-cell metabolism (Fig. 5D and H). Generally, the effects on intact islets were less pronounced than those in dispersed cells, suggesting that cell–cell coupling may dampen the amplified responses of individual Bcl-2/−/− β-cells. The intermediate augmentation of glucose-induced Ca2+ responses in Bcl-2 heterozygous β-cells (Fig. 5B and C) indicates that the effects were dependent on gene dosage and independent of pathological conditions that limit the number of healthy Bcl-2/−/− mice available for study. Throughout our studies, we did not notice any obvious differences in the viability of Bcl-2/−/− islets or β-cells in culture.

**Genetic ablation of Bcl-xL enhances β-cell glucose responses.** Global deletion of Bcl-xL is embryonically lethal (32), so to assess the specific role of Bcl-xL, we generated tissue-specific, tamoxifen-inducible Bcl-xL knockout (BclxflKo) mice. Bcl-xL was knocked out in β-cells of adult animals as early as 2 to 3 days after tamoxifen administration with no compensatory change in Bcl-2 expression (Fig. 6A). Bcl-xL deletion was not observed in hypothalamus (Fig. 6B). Using BclxBKO islet cells, we confirmed a significant molecular contribution of Bcl-xL to C6-induced Ca2+ responses (Fig. 6C). Like Bcl-2/−/− β-cells, BclxBKO β-cells showed significantly larger glucose-induced Ca2+ and NAD(P)H responses (Fig. 6D, E, and G), whereas Ca2+ responses to KCl were normal (Fig. 6F). These findings support a metabolic basis for the amplified Ca2+ responses, although we did not detect changes at the level of glucose oxidation in bulk cultures of dispersed BclxBKO islet cells (Fig. 6H). A modest tendency toward increases in insulin secretion was seen in BclxBKO islets perfused with 3, 6, and 10 mM glucose and in mice injected with glucose in vivo, but these did not achieve statistical significance (Figs. 6I and 7A). Interestingly, glucose tolerance was nonetheless moderately improved in BclxBKO mice administered 2 g/kg glucose relative to littermate controls (Fig. 7B). The potentiation of β-cell Ca2+ signals at submaximal glucose prompted us to examine the in vivo response to a more moderate glucose challenge (0.5 g/kg), and indeed glucose tolerance was also improved under these conditions (Fig. 7C and D). This was not associated with improved insulin sensitivity (Fig. 7E). Together, our combined findings using chemical inhibitors and islets from knockout mice point to novel roles for both Bcl-2 and Bcl-xL in the fine-tuning of glucose signaling in pancreatic β-cells.

**Bcl-2 and Bcl-xL affect β-cell function independently of Bax and Bak.** Proapoptotic Bax and Bak have been reported to interact with two regulators of mitochondrial physiology: the voltage-dependent anion channel (VDAC) and the adenine nucleotide translocase (ANT) (33–35), but it is not clear if this occurs outside of apoptosis. Given the established roles of Bax and Bak downstream of Bcl-2/Bcl-xL, we asked if they participate in Bcl-2/Bcl-xL regulation of metabolism. Bax−/− or Bak−/− β-cells responded to Bcl-2/Bcl-xL inhibition similar to wild-type β-cells (Fig. 8A). To conclusively exclude the involvement of Bax and Bak, we generated mice lacking both genes in their β-cells (Fig. 8B and C). Islet cells from these Bax−/−Bak−/− double-knockout (DKO) mice responded to Bcl-2/Bcl-xL, inhibition similar to wild-type β-cells (Fig. 8A). Together, these data establish that Bax and Bak do not mediate the effects of Bcl-2 or Bcl-xL in β-cell function and further distinguish the physiological effects of Bcl inhibition from Bax/Bak-dependent apoptotic events.

**DISCUSSION**

The current study was undertaken to examine the physiological roles of endogenous prosurvival Bcl proteins in pancreatic β-cells. Using small-molecule Bcl-2/Bcl-xL antagonists and upon Ca2+ removal. C: Nifedipine blocks ongoing C6-induced Ca2+ influx (n = 14 cells). D: Quantification of nifedipine, diazoxide (Dz), and CCCP-mediated suppression of cytosolic Ca2+ responses in mouse islet cells exposed to C6 or YC137 (n = 3). E: Insulin secretion from dispersed islet cells treated with C6, diazoxide, and/or tolbutamide (Tolb) (n = 5). P < 0.05 vs. 3 mM glucose control. F: Percentage of PI-positive mouse islet cells following culture with C6 with or without the presence of nifedipine or Dz (n = 3). G: Reversible inhibition of C6-induced Ca2+ signaling in mouse islet cells by sodium azide (NaN3) and F: Relative changes in ∆Ψm of primary mouse β-cells exposed to stimulatory glucose and the Bcl-2 antagonist C6. In panel H, glucose was added prior to C6. The black line is representative of 38 cells exposed to 80 μM, and the superimposed red line is representative of 15 cells responding to a shorter stimulation with 20 mM glucose. Panel I illustrates the addition of glucose during the C6-induced response (representative of 17 cells). Loss of rhodamine 123 fluorescence indicates mitochondrial hyperpolarization. J: MIN6 β-cell expressing the mitochondrial FRET-based Ca2+ sensor mtD3cpv and examples of mitochondrial Ca2+ fluctuations induced by glucose or Bcl-2/Bcl-xL inhibition (n = 29 cells at 40 mM/L C6; n = 34 cells at 80 mM/L C6). K: Change in the cellular ATP-to-ADP ratio of MIN6 β-cells following 30 min culture in stimulatory 20 mM/L glucose (20G) or in 3 mM/L glucose with 60 mM/L C6, relative to 3 mM/L glucose alone. The depletion seen with CCCP reflects the metabolic pool of ATP (n = 3 cultures; *P < 0.05, **P < 0.001 vs. 3 mM/L glucose; n.s., not significant). Data are mean ± SEM. Basal glucose is 3 mM/L in all experiments. (A high-quality color representation of this figure is available in the online issue.)
islets from KO mice, we provide the first loss-of-function evidence that Bcl-2 and Bcl-xL acutely affect mitochondrial function, Ca²⁺ homeostasis, and insulin secretion. A previous report described apoptosis sensitivity in mice in which Bcl-xL was deleted in embryonic β-cells, but did not provide information on glucose homeostasis or β-cell physiology (5).

**FIG. 5.** Loss of Bcl-2 enhances β-cell glucose responses. A: Quantitative PCR (qPCR) quantification of Bcl-2 and Bcl-xL mRNA levels in islets from Bcl-2⁺/- and Bcl-2⁻/⁻ mice relative to Bcl-2⁺/+ littermates (n = 3 mice of each genotype). All data are mean ± SEM. B: Average cytosolic Ca²⁺ levels of dispersed islet cells from littermate Bcl-2⁺/+, Bcl-2⁺/-, and Bcl-2⁻/⁻ mice. Shaded hanging bars represent SEM. C: Incremental area under the curve of Ca²⁺ responses (n = 98 Bcl-2⁺/+ cells, n = 144 Bcl-2⁺/- cells, and n = 147 Bcl-2⁻/⁻ cells from three mice of each genotype; **P < 0.001 Bcl-2⁻/⁻ vs. Bcl-2⁺/+). D: Integrated cytosolic Ca²⁺ responses of Bcl-2⁻/⁻ and Bcl-2⁺/+ β-cells depolarized with 30 mmol/L KCl (n = 87 Bcl-2⁻/⁻ cells and 130 Bcl-2⁺/+ cells from three mice of each genotype). n.s., not significant. E and F: Integrated Ca²⁺ and NAD(P)H autofluorescence increases of intact islets, normalized to Bcl-2⁺/+ control (panel E: n = 16 Bcl-2⁺/+ islets; n = 20 Bcl-2⁻/⁻ islets; n = 21 Bcl-2⁻/- islets; and panel F: n = 16 Bcl-2⁺/+ islets; n = 17 Bcl-2⁻/- islets; n = 17 Bcl-2⁻/- islets; three mice of each genotype; **P < 0.05, ***P < 0.001 vs. Bcl-2⁺/+). G: Insulin secretion profiles of perfused islets from 5–7-week-old Bcl-2⁺/+ and Bcl-2⁻/⁻ mice. H: Quantified area under the curve of insulin secretion profiles in panel G (n = 5; **P < 0.05 vs. Bcl-2⁺/+). a.u., arbitrary units. (A high-quality color representation of this figure is available in the online issue.)
robust than the genetic ablation of either protein individually. It is likely that combined tissue-specific deletion of Bcl-2 and Bcl-x will be required to mimic the effects of the inhibitors on basal β-cell activity and viability. Our work demonstrates new physiological roles for two proteins previously presumed to function mainly in the control of apoptosis.

Sustained cytosolic Ca\(^{2+}\) rises and insulin release following glucose stimulation rely heavily on mitochondrial ATP synthesis, K\(_{ATP}\) channel-dependent β-cell depolarization, and voltage-gated Ca\(^{2+}\) influx (28). In this study, we report that acute coinhibition of antiapoptotic Bcl-2 and Bcl-xL stimulates an identical cascade of events culminating

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**FIG. 6.** Inducible deletion of Bcl-xL enhances β-cell glucose signaling. A: Quantification of Bcl-xL and Bcl-2 mRNA levels by quantitative PCR (qPCR) (n = 3) and Bcl-xL protein by Western blot (n = 6) in islets from tamoxifen injected Bcl-x\(^{lox/lox}\)Pdx1-CreER (Bcl-x KO) mice relative to islets from tamoxifen injected littermate Bcl-x\(^{lox/lox}\) (Bcl-x WT) mice (data are mean ± SEM; *P < 0.05). B: qPCR quantification of Bcl-xL mRNA in hypothalamus from Bcl-x WT and KO mice (n = 3). C: Percentage of Bcl-x WT and βKO islet cells responding to small-molecule Bcl inhibition (n = 5 mice of each genotype; **P < 0.001 vs. Bcl-x WT). D: Average cytosolic Ca\(^{2+}\) responses of Bcl-x KO and WT β-cells stimulated with increasing glucose concentrations (Conc.). Shaded hanging bars represent SEM. E: Incremental area under the curve of Ca\(^{2+}\) responses. F: Integrated Ca\(^{2+}\) responses of Bcl-x KO and Bcl-x WT β-cells depolarized with 30 mmol/L KCl (n = 66 Bcl-x WT cells; n = 73 Bcl-x KO cells; three mice per genotype; **P < 0.001). G: Integrated NAD(P)H increases of intact islets following glucose stimulation (n = 11 islets, two mice of each genotype; *P < 0.05). H: Glucose oxidation rates in cultures of dispersed Bcl-xL WT and KO islet cells (n = 4). I: Insulin secretion from perifused Bcl-xL WT and KO islets (n = 5). a.u., arbitrary units. (A high-quality color representation of this figure is available in the online issue.)
in insulin secretion. Moreover, genetic deletion of Bcl-2 or Bcl-xL increased the in vitro β-cell responses to glucose and improved in vivo glucose tolerance of the islet-specific Bcl-xL KO mice. Our experiments suggest that this involves amplification of β-cell glucose metabolism and thus that Bcl-2 and Bcl-xL restrict β-cell metabolic activity. Our findings conceptually agree with a previous study in which mice overexpressing Bcl-xL 10-fold under the control of the rat insulin promoter exhibited impaired β-cell oxidative metabolism and glucose intolerance (9). Several groups have overexpressed Bcl-2 in pancreatic islets as part of efforts to block apoptosis, but we are only aware of a few studies that examined the impact on β-cell function, and these reported no impairment of insulin secretion (36,37). This could be interpreted as evidence for a saturation effect whereby excess levels of Bcl-2 protein do not negatively affect the stoichiometry of complexes associated with the metabolic machinery. Alternatively, the lower fraction of mitochondria-localized Bcl-2 relative to Bcl-xL (Fig. 3) might require that correspondingly larger amounts of Bcl-2 are expressed to achieve detectable metabolic suppression.

FIG. 7. Improved glucose tolerance in Bcl-x βKO mice. A: In vivo insulin secretion following intraperitoneal injection of 2 g/kg glucose in 10–12-week-old Bcl-x WT and βKO littermate mice (n = 5). B and C: Intraperitoneal glucose tolerance tests of Bcl-x βKO and WT mice using 2 and 0.5 g/kg glucose doses (n = 7 and n = 8, respectively; *P < 0.05). D: Area under the curve analysis of glucose profiles in panels B and C. E: Insulin tolerance test of Bcl-x WT and βKO mice (n = 5). (A high-quality color representation of this figure is available in the online issue.)
We considered that antagonizing Bcl-2/Bcl-xL might free Bax and/or Bak to indirectly activate mitochondria, possibly by affecting ANT or VDAC (33–35). We tested and eliminated this possibility using Bax KO, Bak KO, and Bax/Bak DKO islet cells. Work from the Danial group (3,38) is consistent with an alternative indirect model whereby Bcl-2 and/or Bcl-xL might sequester Bad and limit its promotion of glucokinase activity. Bcl-2 and Bcl-xL have not been detected in the Bad/glucokinase complex, but reducing their binding to Bad might release this brake on b-cell metabolism from afar. In preliminary studies in MIN6 cells, Bcl inhibition did not change Bad levels or phosphorylation within 60 min, the timescale corresponding to the acute Ca2+ signals and metabolic effects. Preliminary studies in Bcl-xL KO islets revealed 58% increase in serine 155 phosphorylation, with no significant effects on the phosphorylation of Bad at serine 136 or serine 112. Thus, phosphorylation-dependent functions of Bad do not appear acutely involved in the effects of Bcl antagonism. However, a more chronic contribution from the Bad/glucokinase axis following chronic Bcl protein loss remains a possibility that might promote the amplification of insulin secretion in stimulatory glucose that is not apparent acutely following inhibition with small molecules.

Our data also allow for a model whereby Bcl proteins directly affect mitochondrial proteins in the b-cell, provided such interactions are changed by Bcl antagonists. Studies of the antiapoptotic activities of Bcl-2 and Bcl-xL

**FIG. 8. Effect of Bcl antagonism in Bax, Bak, and Bcl-xL-deficient islet cells.** A: Percentage of islet cells responding to Bcl antagonism in preparations from Bax−/− (left), Bak−/− (right), and their wild-type control mice (n = 3 mice). Data are mean ± SEM. Basal glucose was 3 mmol/L in all experiments. B: Western blot demonstrating global Bak deficiency and islet specific Bax knockout in tamoxifen-injected Bak−/−:Baxlox/lox:Pdx1-CreER (Bax-Bak βDKO) mice relative to tamoxifen-injected Bak−/−:Baxlox/lox and C57BL6/J (C57) mice. C: Bak protein levels were reduced by 85% in Bak-Bak βDKO islets (n = 6; **P < 0.001 vs. Bak−/−:Baxlox/lox). D: Comparable Bcl inhibitor-induced Ca2+ responses in groups of Bak−/−:Baxlox/lox and Bak-Bak βDKO islet cells. E: Percentage of Bak-Bak βDKO and Bak−/−:Baxlox/lox islet-cells responding to Bcl inhibition (n = 3 mice of each genotype). (A high-quality color representation of this figure is available in the online issue.)
have reported that they can interact with mitochondrial ANT and VDAC via their BH4 domains (35). We have found that a cell-permeant Bcl-xL BH4 domain peptide triggers cytosolic and mitochondrial Ca2+ fluctuations in β-cells (D.S.L and J.D.J. unpublished observations). This could result from direct mitochondrial actions of the BH4 domain and/or ER Ca2+ release (39). Of note, it was recently reported that Bcl-xL can lower acetyl-CoA levels independently of Bax and Bak (40). Another study suggested that Bcl-xL suppresses O2 consumption, although promoting ATP synthesis in neurons by interacting with the mitochondrial F1F0 ATPase (8), indicating that Bcl-xL can have opposing metabolic effects in a cell. Conceivably, changes in the relative contributions of these effects may shape the net metabolic impact of Bcl-xL in a given cell type and might complicate the analysis of complex mechanisms such as insulin secretion that involve multiple metabolic pathways.

Our finding that antiapoptotic Bcl-2 family proteins can modulate β-cell function has intriguing implications for our understanding of the pathophysiology of diabetes. The signal transduction machinery of β-cells is optimized for maximal delivery of glycolytic intermediates for oxidative phosphorylation (41). However, β-cells are remarkably sensitive to the deleterious effects of reactive oxygen species (42). Metabolic suppression may provide a means by which Bcl proteins protect pancreatic β-cells against metabolic stress. One might also speculate that the reduction in Bcl-2 and Bcl-xL seen under prodiabetic conditions (4,43–45) can affect β-cell function. In this regard, it is noteworthy that insulin hypersecretion is an early marker of human diabetes (46), and chronic hyperinsulinemia a persistent feature of diabetic animal models, including the Zucker Diabetic Fatty rat (47), which has 70% less Bcl-2 protein (48). In extension of this, our results suggest caution may be prudent in efforts to treat diabetes by augmenting β-cell metabolic flux using agents such as glucokinase activators (49).

In summary, we demonstrate novel roles of endogenous antiapoptotic Bcl proteins in the physiology of pancreatic β-cells. Specifically, our data suggest that endogenous Bcl-2 and Bcl-xL suppress the β-cell response to glucose. Our findings add to emerging evidence that places Bcl family mechanisms such as insulin secretion that involve multiple metabolic pathways.

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D.S.L. designed experiments, performed research, and wrote the manuscript. S.A.W., S.B.W., and V.S. performed research, contributed to discussion and reviewed and edited the manuscript. F.T. performed research. X.H. performed research. M.F.A. contributed to discussion and reviewed and edited manuscript. J.D.J. designed research and wrote the manuscript. J.D.J. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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