Glucose induces pancreatic islet cell apoptosis that requires the BH3-only proteins Bim and Puma and multi-BH domain protein Bax

Running title: Glucose-induced beta cell apoptosis

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**Objective:** High concentrations of circulating glucose are believed to contribute to defective insulin secretion and beta-cell function in diabetes and at least some of this effect appears to be caused by glucose-induced beta-cell apoptosis. In mammalian cells, apoptotic cell death is controlled by the interplay of pro-and anti-apoptotic members of the Bcl-2 family. We investigated the apoptotic pathway induced in mouse pancreatic islet cells after exposure to high concentrations of the reducing sugars ribose and glucose as a model of beta cell death due to long-term metabolic stress.

**Research design and methods:** Islets isolated from mice lacking molecules implicated in cell death pathways were exposed to high concentrations of glucose or ribose. Apoptosis was measured by analysis of DNA fragmentation and release of mitochondrial cytochrome c.

**Results:** Deficiency of IL-1 receptors or Fas did not diminish apoptosis, making involvement of inflammatory cytokine receptor or death receptor signalling in glucose-induced apoptosis unlikely. In contrast, overexpression of the pro-survival protein Bcl-2 or deficiency of the apoptosis initiating BH3-only proteins Bim or Puma or the downstream apoptosis effector Bax, markedly reduced glucose or ribose-induced killing of islets. Loss of other BH3-only proteins Bid or Noxa, or the Bax-related effector Bak had no impact on glucose-induced apoptosis.

**Conclusions:** These results implicate the Bcl-2 regulated apoptotic pathway in glucose-induced islet cell killing and indicate points in the pathway at which interventional strategies can be designed.
Type 2 diabetes develops when insulin resistant subjects develop pancreatic beta-cell dysfunction (1-3). Progressive beta-cell dysfunction results in insufficient insulin secretion to compensate for insulin resistance. The relative contribution of a decrease in beta cell mass versus a functional defect in insulin secretion towards the overall morbidity remains unclear. Using human pancreatic tissue from autopsies, Butler et al showed that there was an approximately 60% reduction in beta cell mass in type 2 diabetes patients compared to non-diabetic controls, and this was attributed to a 10-fold or 3-fold increase in beta cell apoptosis in type 2 diabetes patients who were lean or obese respectively (4). Although the cause of this apoptosis is not yet clear, glucose, saturated fatty acids, islet amyloid polypeptides and IL-1β have all been implicated, and these molecules are toxic to beta cells and beta cell lines in vitro.

High concentrations of glucose can cause beta cell apoptosis and, in addition to a potential role in beta cell dysfunction in type 2 diabetes (2), high circulating glucose concentrations may also contribute to destruction of the remaining beta cells at the time of diagnosis of type 1 diabetes or when the beta cell mass in an islet transplant is marginal. Beta cell apoptosis attributed to glucose toxicity has been observed in several animal models of type 2 diabetes including the Psammomys obesus desert gerbil (5), the Zucker diabetic fatty rat (6), and the domestic cat (7). Isolated islets from P. obesus are susceptible to glucose-dependent DNA fragmentation (5).

Several mechanisms for glucose-induced islet toxicity have been proposed. In human islets it has been suggested that glucose induces intra-islet production of IL-1β, leading to NF-κB activation, Fas up-regulation and beta cell apoptosis as a consequence of engagement by FasL, expressed on neighboring beta cells (8-10). However, these findings could not be reproduced in other studies (11, 12), leading to alternative mechanisms being suggested. Beta cells are vulnerable to endoplasmic reticulum (ER) stress due to their enormous demand to synthesize and secrete insulin, and high glucose levels may exacerbate this (reviewed in (13)). High concentrations of reducing sugars were also reported to induce intracellular peroxides that elicit beta cell death (14). The expression of intrinsic antioxidant enzymes is normally quite low in beta cells (15), and adenoviral overexpression of Gpx-1 prevented glucose-induced apoptosis (14). Glucose induced expression of the pro-apoptotic factor thioredoxin-interacting protein, which inhibits the redox-active protein thioredoxin, and when overexpressed, induces caspase 3-dependent beta cell apoptosis (16). Glucose also promoted degradation of cyclic AMP-responsive element binding protein (CREB) by the ubiquitin-proteasome pathway leading to beta cell apoptosis (17).

In mammalian cells two distinct pathways control apoptosis, the “death receptor” (also called “extrinsic”) and the “mitochondrial” (also called “intrinsic” or “Bcl-2 regulated”) pathways. In the intrinsic pathway, the eight BH3-only proteins (Bim, Bid, Bad, Puma, Noxa, Hrk, Bik and Bmf) initiate apoptosis signalling by binding to the Bcl-2-like pro-survival proteins (Bcl-2, Bcl-xL, Bcl-w, Mcl-1 and A1), thereby releasing Bax and/or Bak to promote loss of mitochondrial outer membrane potential, cytochrome c release and activation of the caspase cascade (18). Direct activation of Bax and/or Bak by certain BH3-only proteins has also been proposed (19). Exposure of human islets to 16.5mM glucose in vitro for 5 days resulted in up-regulation of Bid and Bad and down-regulation of Bcl-xL resulting in the death of beta cells (20). We have shown that
Bid deficiency prevents Fas ligand-induced beta cell apoptosis (21), whereas Bcl-2 overexpression protects beta cells from a range of apoptotic stimuli including treatment with pro-inflammatory cytokines, Fas ligand or staurosporine (21, 22). Mice deficient in Bad were shown to have impaired glucose homeostasis due to defective insulin secretion in response to glucose stimulation (23) but this was not due to abnormalities in beta cell apoptosis. Bim is transcriptionally up-regulated by C/EBP homologous protein (CHOP) and required for ER stress-induced apoptosis in a range of cell types (24). Puma has also been implicated in ER stress-induced apoptosis in certain cell types (25). The requirement for these molecules has not yet been examined in beta cells.

Understanding how glucose toxicity triggers islet cell apoptosis is likely to shed new light on mechanisms of beta-cell loss in diabetes and may therefore pave the way to improved therapeutic intervention. We have investigated the pathways of apoptosis induced in islet cells by high concentrations of reducing sugars. We found that IL-1R and Fas are dispensable for this process, as was Bid that we have shown is required for extrinsic pathway apoptosis in islet cells. In contrast, Bcl-2 over-expression, loss of the BH3-only proteins Bim or Puma or loss of the multi-BH domain protein Bax markedly protected islets from glucose toxicity.

**RESEARCH DESIGN AND METHODS**

**Mice:** IL-1 receptor (IL-1R−/−) deficient mice, generated on a mixed C57BL/6x129SV background (using 129SV-derived ES cells), were obtained from Dr. M. Labow (Roche) and backcrossed onto the C57BL/6 background for 8 generations (26). C57BL/6 mice and Fas deficient Fas−/− mice on a C57BL/6 genetic background were obtained from the Walter and Eliza Hall Institute animal breeding facility (Kew, Victoria, Australia). Mice globally deficient for bim, puma, noxa, bid, bax or bak have been previously described (27-31). Mice deficient for puma, noxa or bid were generated on an inbred C57BL/6 background using C57BL/6-derived ES cells. Mice deficient for bim, bax or bak were originally generated on a mixed C57BL/6x129SV genetic background using 129SV-derived ES cells and were backcrossed for >10 generations onto the C57BL/6 background. H-2bm RIP-Bcl-2 transgenic mice which express human Bcl-2 in beta cells under control of the rat insulin promoter have previously been described (22). All animal experiments were approved by the institutional animal ethics committee.

**Reagents:** D-glucose, L-glucose (used at 33.3 mM), D-ribose and L-ribose (used at 50 mM) were purchased from Invitrogen (Gibco products Invitrogen Corporation, Grand Island, New York USA) and Sigma-Aldrich (St Louis, MO) respectively. The concentration of D-glucose or D-ribose and the time of incubation were titrated to determine optimal culture conditions. The concentrations we used were similar to those used in previously published reports (9, 14, 32). The pan-caspase inhibitor qVD.oph (Enzyme Systems Products, Livermore, California, USA) was used at 50 µM. Recombinant murine γ-interferon (IFNγ) was obtained from Genentech (San Francisco, CA) and used at 100 units/ml. Human recombinant interleukin-1β from R&D Systems (Minneapolis, MN) was used at 150 units/ml.

**Preparation of islets:** Islets of Langerhans were isolated by collagenase P (dissolved in HBSS containing 2mM Ca²⁺ and 20 mM HEPES) digestion and density gradient centrifugation as described previously (33). Islets were washed, hand-picked and cultured overnight at 37°C in 5% CO₂ in CMRL medium-1066 (Gibco products Invitrogen Corporation) supplemented with 100 U/mL penicillin, 100 µg/mL
streptomycin, 2 mM glutamine and 10% foetal calf serum (FCS) (JRH Biosciences, Kansas, USA) (referred to below as complete CMRL). We did not observe any differences in number or size of islets isolated from mice deficient in Bcl-2 family genes, suggesting that at a global level, deficiency of these genes does not affect islet development (data not shown).

**DNA fragmentation assay:**
Uniformly sized islets (excluding very large or necrotic islets) were handpicked into 3.5 cm Petri dishes containing 1.1 mL of complete CMRL. Islets were then cultured with the appropriate stimuli to induce apoptosis. At the end of the culture period, non-attached cells and islets were transferred into polypropylene tubes and washed in PBS. Islets were then dispersed with trypsin (0.1 mg/mL bovine trypsin (Calbiochem) and 2 mmol/L EDTA in PBS) for 5 min at 37°C. Islets were mechanically dispersed using a pipette, washed in PBS, and allowed to recover in complete CMRL medium for 1 h at 37°C in 5% CO\textsubscript{2}. Cells were then washed in PBS and resuspended in 250 µL of hypotonic buffer containing 50 µg/mL propidium iodide (Sigma-Aldrich), 0.1% sodium citrate, and 0.1% Triton X-100 which stains nuclear DNA. The cells were then analyzed on a FACSCalibur (Becton Dickinson, Franklin Lakes, New Jersey, USA) using the FL3 channel. Cells undergoing apoptosis were identified by their apparent sub-diploid DNA content as previously described (34).

**Cytochrome c release assay:** Islets were dispersed into single cells using trypsin, and cytochrome c release was measured using the method previously described (35). Briefly, cells were permeabilized in 100 µL digitonin buffer (80 mmol/L KCl, 50 ng/mL digitonin and 1 mmol/L EDTA in PBS) for 2 min on ice. Permeabilized cells were fixed with 4% paraformaldehyde in PBS for 20 min at room temperature, washed in PBS and incubated in blocking buffer (3% BSA and 0.05% Saponin in PBS) for 1 h. Cells were then stained overnight at 4°C with mouse anti-cytochrome c antibody (Clone 6H2B4, BD Pharmingen) followed by staining with phycoerythrin-conjugated sheep anti-mouse IgG antibodies (Silenus, Hawthorn, Australia) in blocking buffer for 1 h at room temperature. Cells were analyzed on a FACSCalibur. The control samples had a typical background between 5-10% cytochrome c release. This is consistent with published reports using this method (35).

**Real-time qRT-PCR analysis:** RNA was prepared using the RNeasy Kit (Qiagen). First strand cDNA was prepared from 0.1-0.2 µg RNA using the High Capacity RNA-to-cDNA Master Mix (Applied Biosystems). Real-time PCR was performed using the ABI Prism 7900 (Applied Biosystems) and the Power SYBR Green PCR Master Mix (Applied Biosystems) in 15 µl reaction volumes. Data analyses were performed with the CT method using actin as an internal control. qRT-PCR was performed using the forward and reverse primers in Table 1.

**Western blotting:** Islets were incubated for 4 days with cytokines and/or 33.3 mM glucose or 50 mM ribose, washed with PBS and lysed in 10 µl of lysis buffer (20 mM Tris/HCl pH 7.4, 135 mM NaCl, 1.5 mM MgCl\textsubscript{2}, 1 mM EGTA, 1% Triton X-100, 1 x protease inhibitor cocktail (Sigma), and 1 x phosphatase inhibitor cocktail (Sigma)). Samples were separated by SDS-PAGE and transferred to nitrocellulose using standard procedures. Western blotting was performed with anti-NOS2 Ab (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Bim Ab (clone 3C5, Alexis, Plymouth Meeting, PA), anti-Puma Ab (ProSci Inc, Poway, CA) and anti-Actin Ab (Santa Cruz Biotechnology, Santa Cruz, CA) followed by horseradish peroxidase (HRP)-conjugated secondary antibodies (Silenus Laboratories, Hawthorn, Australia) and detection with ECL Western blotting detection system (GE Healthcare).
Nitrite determination: Nitrite was detected in the cultures by mixing 100 µl supernatant with 100 µl Greiss reagent (36). Absorbances were read at 540 nm, and nitrite concentrations were calculated using a sodium nitrite standard curve.

Statistical analysis: Statistical analyses of data were performed using GraphPad Prism (GraphPad Software, San Diego, California, USA). All data shown as bar graphs are represented as means ± SEM. Data were analysed by one-way or two-way ANOVA with Bonferroni’s post test for comparison of multiple columns, or by two-tailed paired t-tests.

RESULTS

Abnormally high concentrations of glucose or ribose induce DNA fragmentation in islet cells: Islets from wild type C57BL/6 mice were exposed to 33.3 mM glucose or 50 mM ribose in vitro, and DNA fragmentation was examined by FACS analysis as a measure of apoptosis. Wild-type islets cultured in high concentrations of D-glucose for 6 days displayed a significant increase in DNA fragmentation compared to islets cultured in medium containing only 5.6 mM glucose (Fig. 1A), or those cultured in medium with 33.3 mM L-glucose (osmolarity control). D-Ribose mimics the effects of glucose exposure (14) and it killed islet cells more rapidly, and to a greater extent than high glucose (Fig. 1A).

To confirm that glucose toxicity triggered apoptosis in islet cells, we examined release of cytochrome c from the mitochondria, an event that occurs upstream of inter-nucleosomal DNA fragmentation. High glucose concentrations promoted release of cytochrome c from the mitochondria (Fig.1B). Consistent with the analysis of DNA fragmentation, high concentrations of ribose triggered mitochondrial cytochrome c release more rapidly and to a greater extent than treatment with glucose (Fig. 1B). The pan-caspase inhibitor qVD.oph significantly inhibited ribose-induced islet cell DNA fragmentation (Fig. 1C), confirming that cell killing occurred through a caspase-dependent apoptotic process.

Islets deficient in IL-1 receptors or Fas are not protected from glucose induced DNA fragmentation: Previous studies have suggested that high glucose concentrations result in IL-1β production by beta cells, leading to Fas up-regulation and autocrine or paracrine FasL-Fas-induced apoptosis signalling (9, 10). To determine the contribution of this proposed cell death pathway to glucose-induced beta-cell killing, DNA fragmentation was examined in islets from mice lacking IL-1 receptors (IL-1R−/−) or functional Fas (Fas lpr/lpr). In response to culture in 50 mM ribose or 33.3 mM glucose, DNA fragmentation was comparable between islets from wild-type, IL-1R−/− or Fas lpr/lpr mice (Fig. 2A). In addition, loss of the BH3-only protein Bid, which we have shown to be essential for death receptor-mediated islet cell apoptosis (21), also failed to protect islets from ribose or glucose toxicity (Fig. 2A). These results suggest that IL-1R and Fas are not involved in glucose/ribose induced killing of murine islets.

Incubation of mouse islets with recombinant IL-1β and IFNγ leads to the induction of iNOS expression, NO2 production and DNA fragmentation. If high concentrations of glucose induce functional concentrations of intra-islet IL-1β production, then these downstream effects should be measurable. Therefore we tested whether 33.3 mM glucose together with IFNγ could induce iNOS expression, NO2 production and DNA fragmentation. Our results show that although recombinant IL-1β and IFNγ were able to induce iNOS expression, as well as a significant increase in NO2 production and DNA fragmentation, no iNOS expression, NO2 production or DNA fragmentation were observed in islets cultured in 33.3 mM
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... glucose in the presence of IFNγ (Fig 2 B-D). Together these data rule out a role for IL-1 in glucose toxicity of mouse islets.

**Bcl-2 over-expression protects islet cells from glucose or ribose toxicity-induced cytochrome c release and DNA fragmentation:** Disruption of the mitochondria and release of cytochrome c is controlled by the Bcl-2 family of proteins (37). Therefore if cytochrome c release represents a critical point in toxicity of islets to high concentrations of ribose or glucose, over-expression of pro-survival Bcl-2 family members, such as Bcl-2 itself, should inhibit this apoptotic pathway. We therefore treated islets over-expressing Bcl-2 in beta cells and control islets from non-transgenic animals with high concentrations of ribose or glucose and compared their susceptibility to apoptosis. Indeed, over-expression of Bcl-2 reduced cytochrome c release from the mitochondria and significantly inhibited DNA fragmentation triggered by exposure to 50 mM ribose (Fig. 3A,B). Similarly, over-expression of Bcl-2 substantially reduced cytochrome c release and DNA fragmentation in islets cultured in medium containing 33.3 mM glucose (Fig. 3C, D).

**Loss of Bax but not Bak protects islets from glucose or ribose induced DNA fragmentation:** The multi-BH domain pro-apoptotic Bcl-2 family members Bax and Bak are essential for mitochondrial outer membrane permeabilization (MOMP) during apoptosis signaling and play essential and in many cell types overlapping roles in the activation of the execution phase of apoptosis (30). We tested whether loss of either of these proteins would result in protection from high concentrations of ribose or glucose. Bax-deficient islets were resistant to ribose or glucose-induced cytochrome c release and DNA fragmentation, whereas islets from Bak-deficient mice were normally sensitive (Fig. 4). This result demonstrates that Bax plays a critical role in glucose or ribose-induced islet killing.

**Individual loss of the BH3-only proteins Bim or Puma inhibits glucose or ribose toxicity-induced mitochondrial cytochrome c release and DNA fragmentation:** The observation that Bcl-2 over-expression or loss of Bax protects islet cells from glucose toxicity demonstrated that the mitochondrial pathway of apoptosis is involved, and indicated that it is initiated by members of the BH3-only subgroup of the Bcl-2 family. We therefore isolated islets from a panel of BH3-only deficient mice (Bim-/-, Noxa-/-, Puma-/- or Bid-/-) to examine their sensitivity to glucose or ribose toxicity. Deficiency of either Bim or Puma reduced cytochrome c release (Fig. 5A) and DNA fragmentation (Fig 5B) from the mitochondria to a similar extent as over-expression of Bcl-2. In contrast, loss of the BH3-only proteins Noxa or Bid had no impact (Fig. 5A-C).

BH3-only proteins often cooperate to induce apoptosis; for example it has been shown that combined loss of Bim and Puma protects lymphoid and myeloid cells more potently against a range of apoptotic stimuli such as cytokine deprivation, than loss of either BH3-only protein alone (38). Because apoptosis induced by ribose or glucose toxicity was only partially inhibited by loss of either Bim or Puma, we examined the possibility that these proteins cooperate in glucose or ribose-mediated apoptosis. Remarkably, the extent of apoptosis induced by 50 mM ribose (Fig. 5D) or 33.3 mM glucose (Fig. 5E) in islets deficient in both Bim and Puma was not significantly above the background level observed in islets cultured in control medium, and significantly lower than apoptosis seen in islets lacking only Bim or Puma or those overexpressing Bcl-2 (compare Fig 5B and Fig 5D). These data demonstrate that Bim and Puma are both...
activated and cause glucose or ribose toxicity-induced apoptosis in islet cells.

**Glucose and ribose toxicity cause up-regulation of Puma and Bax mRNA in islet cells:** The ability of ribose or glucose to induce expression of Bim, Puma, Bax and Bak was measured by quantitative RT PCR. After 48 h exposure to 50 mM ribose, expression of puma and bax increased 4-fold (Fig. 6A-C). Very low levels of Bak were detectable, and these did not change after exposure to glucose or ribose (data not shown). Incubation with 5 µM thapsigargin, which induces apoptosis of islets (39), induced a 2-7-fold increase in expression of puma, bax (Fig. 6A-C) and bak (data not shown), but not bim. Bim expression remained unchanged, even though this basal level of bim was clearly sufficient to cause apoptosis in response to ribose incubation. We used PCR primers that detect BimL and BimEL, but not BimS, so we can not exclude the possibility that BimS is playing a role. We also determined protein expression by Western blotting. Consistent with the qrtPCR data, we observed an increase in Puma expression after incubation with 33.3 mM glucose or 50 mM ribose, but no change in Bim expression or phosphorylation (Fig. 6D). We therefore surmise that the basal level of Bim, or a modification that is not detectable by Western blotting is critical for glucose toxicity induced killing of beta cells.

**DISCUSSION**

We have exposed pancreatic islets to high concentrations of glucose or ribose in vitro as a model of the long-term metabolic stress on beta cells observed in insulin resistance and diabetes. We found conclusive evidence that excessive glucose can cause apoptosis in islet cells by the “mitochondrial” (also called “intrinsic” or “Bcl-2-regulated”) pathway. Glucose or ribose caused release of cytochrome c and DNA fragmentation that are both hallmarks of apoptosis mediated by the mitochondrial pathway of cell death (40). The ability of over-expression of the anti-apoptotic protein Bcl-2 to partially inhibit beta cell death confirms the involvement of this pathway. Altered expression of Bcl-2 family genes has also been observed after exposure of human islets to high concentrations of glucose, also implicating the intrinsic pathway in glucose toxicity (17, 20).

Mitochondrial outer membrane disruption and effector caspase activation are initiated by activation of the pro-apoptotic BH3-only Bcl-2 family members (37, 41). BH3-only family members are activated in a death stimulus- and cell type-specific manner, and the ability to group stimuli that trigger apoptosis by the same pathway may help understand the mechanisms of cellular responses (42). We found that glucose and ribose toxicity-induced apoptosis requires the BH3-only proteins Bim and Puma and the multi-BH domain protein Bax whereas deficiency of other pro-apoptotic proteins, including Bid and Bak, had no impact. In previous studies that examined processes that have been implicated in beta-cell apoptosis in type 1 diabetes, including those triggered by perforin/granzyme B, FasL and TNF, the BH3-only protein Bid was required (21, 43). Therefore glucose and ribose toxicity trigger a distinct apoptotic cascade in islet cells to that triggered by either perforin plus granzyme B or death receptor ligation. This makes it unlikely that high concentrations of glucose kill murine islet cells by the death receptor pathway or a process involving IL-1R signalling. Moreover, Bcl-2 over-expression or caspase inhibition provide minimal protection from cytokine-mediated islet cell death (44, 45). This adds further weight to the notion that this pathway and glucose toxicity-induced apoptosis are distinct.

Both the ER stress and oxidative stress pathways have been associated with the toxic effects of glucose and ribose on islets (13,
Both of these stress pathways have also been reported to activate BH3-only proteins, particularly Bim and Puma, in other cell types. In certain epithelial cells, thymocytes and macrophages, treatment with thapsigargin or tunicamycin caused ER stress-mediated apoptosis that required the BH3-only protein Bim (24). In contrast, in neurons Puma was found to be critical for apoptosis induced by oxidative or ER stress (25, 47). Our results demonstrate that Bim and Puma have critical overlapping roles in glucose toxicity induced killing of islets. This is reminiscent of the finding that combined loss of these two BH3-only proteins protects lymphoid cells against a range of death stimuli, such as cytokine deprivation, more potently than loss of either alone (38). It is possible that glucose toxicity induces Bim in islet cells predominantly by triggering ER stress. In other cell types, ER stress-induced activation of the transcriptional activator CHOP activates Bim, which is essential for ER stress-induced apoptosis (24). Since we found no up-regulation of bim mRNA in islets exposed to high concentrations of glucose or ribose, we surmise that CHOP may be critical for the basal level of bim expression. This may relate to the fact that due to the high demand for protein synthesis and secretion, beta cells are in a constant state of ER stress. Although we were unable to detect evidence of post-translational modification of Bim by Western blotting, we can not exclude the possibility that glucose and ribose toxicity activate Bim post-translationally. The mechanisms by which glucose or ribose toxicity activates Puma are presently not clear, but they may also involve the ER stress pathway.

Perhaps surprisingly, we found that loss of Bax but not loss of Bak protects islet cells from glucose toxicity. This is reminiscent of another study with beta cells, and one with neuronal cells which both found that Bax but not Bak is critical for their Myc-induced or oxidative stress-induced apoptosis, respectively, although both proteins are expressed (47, 48). In the case of glucose or ribose toxicity-induced islet killing, it remains possible that Bak does cooperate with Bax in this pathway to apoptosis since loss of Bax afforded considerably less protection than combined loss of Bim and Puma.

In conclusion we have shown that high concentrations of glucose and ribose cause apoptosis mediated by the mitochondrial pathway in islets. This occurs by specific pathways distinct from those involved in cytokine or death receptor-mediated apoptosis. There is increasing evidence that beta cell mass and beta cell susceptibility to apoptosis are key determinants in transition from insulin resistance to diabetes (49), and in mouse models, blocking islet cell apoptosis can prevent type 2 diabetes (50, 51). Identification of Bim, Puma and Bax as critical mediators in glucose-induced beta cell killing pinpoints possible targets for therapeutic intervention in this disease.

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REFERENCES

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### Table 1. PCR primers used for gene expression analysis

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**FIGURE LEGENDS**

**Figure 1:** High concentrations of glucose or ribose induce DNA fragmentation, cytochrome c release and caspase activation in islets.

100-200 islets from wild-type mice were cultured in complete medium containing 5.5 mM D-glucose (control), or were exposed to 33.3 mM D- or L-glucose or 50 mM D- or L-ribose. (A) DNA fragmentation was measured by flow cytometry after culture for 6 days in glucose or 4 days in ribose. i) Representative FACS profiles are shown ii) Data represent islets from a minimum of 5 individual mice per group. Statistical significance; *p<0.0001 compared with control (one-way ANOVA). (B) Cytochrome c release was measured by flow cytometry after 4 days’ incubation in control medium or glucose, or 3 days in ribose. i) Representative FACS profiles are shown. ii) Data represent islets from 4-8 individual mice per group. Statistical significance; *p<0.0001 **p<0.01 compared with control (one-way ANOVA). (C) DNA fragmentation of islet cells after incubation for i) 4 days in ribose or ii) 6 days in glucose in the presence or absence of the caspase inhibitor qVD.oph was measured by flow cytometry. Data represent islets from 4-5 individual mice per group. Statistical significance; *p<0.0001 compared with ribose or glucose treatment (one-way ANOVA).

**Figure 2:** IL-1R or Fas are not required for glucose or ribose-induced islet cell apoptosis.

(A) 200 islets from wild-type, *IL-1R*^-/-^, *Fas*^lpr/lpr^ or *bid*^-/-^ mice were cultured for 6 days in control medium or medium containing 33.3 mM glucose or for 4 days in medium containing 50 mM ribose. DNA fragmentation was measured by flow cytometry. No significant differences were observed between mice of the different genotypes p= 0.1403 (one-way ANOVA). Data are representative of at least three independent experiments using a minimum of 3 individual mice of each genotype. (B-D) Islets were cultured for 7 days in control medium or 33.3mM with cytokines added in the last 4 days. (B) Western blotting for iNOS and β-actin as a loading control. (C) NO$_2^-$ concentration in culture supernatant was determined. (D) DNA fragmentation was measured by flow cytometry. No significant difference in NO$_2^-$ production or DNA fragmentation was observed between islets cultured with glucose and those cultured with glucose + IFNγ. The data in B-D represent islets from three independent experiments.

**Figure 3:** Bcl-2 over-expression protects islets from glucose or ribose-induced apoptosis.

100 islets from wild-type or RIP.Bcl-2 transgenic mice were cultured in control medium or medium containing 50 mM ribose or 33.3 mM glucose. (A) Cytochrome c release was measured by flow cytometry after 3 days of culture. Data represent islets from 5 individual mice per genotype. Statistical significance; *p<0.0001 compared with wild-type islets in 50 mM ribose (one-way ANOVA). (B) DNA fragmentation was measured by flow cytometry after culture for 4 days with 50 mM ribose. Data represent islets from 5-7 individual mice per genotype. Statistical significance; **p<0.001 compared with wild-type islets in 50 mM ribose (one-way ANOVA). (C) Cytochrome c release was measured by flow cytometry after 4 days of culture. Data represent islets from 4 individual mice per genotype. Statistical significance; *p<0.0001 compared with wild-type islets in 33.3 mM glucose (one-way ANOVA). (D) DNA fragmentation was measured by flow cytometry after 6 days culture in 33.3mM glucose. Data represents islets from 4-6 individual mice per genotype. Statistical significance; #p<0.01 compared with wild-type islets in 33.3 mM glucose (one-way ANOVA).
Figure 4: Loss of Bax but not loss of Bak protects islet cells from ribose or glucose induced cytochrome c release and DNA fragmentation.

100 islets from wild-type, bak\(^{-/-}\) or bax\(^{-/-}\) mice were cultured in control medium or medium containing 50 mM ribose or 33.3 mM glucose. (A) Cytochrome c release was measured by flow cytometry after 3 days of culture in ribose. Data represent islets from 3 individual mice per genotype. Statistical significance; *p<0.0001 compared with wild-type islets in 50 mM ribose (one-way ANOVA). (B) DNA fragmentation was measured by flow cytometry after 4 days of culture in ribose. Data represent islets from 6 individual mice per genotype. Statistical significance; *p<0.0001 compared with wild-type islets in 50 mM ribose (one-way ANOVA). (C) Cytochrome c release was measured by flow cytometry after 4 days of culture in glucose. Data represent islets from 4 individual mice per genotype. Statistical significance; *p<0.0001 compared with wild-type islets in 33.3 mM glucose (one-way ANOVA). (D) DNA fragmentation was measured by flow cytometry after 6 days culture in glucose. Data represent islets from 3 individual mice per genotype. Statistical significance; **p<0.001 compared with wild-type islets in 33.3 mM glucose (one-way ANOVA).

Figure 5: Loss of Bim or Puma protects islets from glucose- or ribose-induced mitochondrial cytochrome c release and DNA fragmentation.

100 islets from wild-type, bim\(^{-/-}\), noxa\(^{-/-}\), puma\(^{-/-}\), bid\(^{-/-}\) or bim\(^{-/-}\)puma\(^{-/-}\) mice were cultured in control medium or medium containing 50mM ribose or 33.3 mM glucose. (A) Cytochrome c release was measured by flow cytometry after 3 days of culture in ribose. Data represent islets from a minimum of 3 individual mice per genotype. Statistical significance; *p<0.0001 compared with wild-type islets in 50 mM ribose (one-way ANOVA). (B) DNA fragmentation was measured by flow cytometry after 4 days of culture in ribose. Data represent islets from a minimum of 3 individual mice per genotype. Statistical significance; *p<0.0001 compared with wild-type islets in 50 mM ribose (one-way ANOVA). (C) DNA fragmentation was measured by flow cytometry after 6 days of culture in glucose. Data represent islets from a minimum of 3 individual mice per genotype. Statistical significance; *p<0.0001 compared with wild-type islets in 33.3mM glucose (one-way ANOVA). (D) DNA fragmentation was measured after 6 days of culture in glucose. Data represent 4-5 individual mice per genotype. Statistical significance; *p<0.0001 compared with wild-type islets in 33.3 mM glucose (one-way ANOVA).

Figure 6: Ribose or glucose induce expression of Puma and Bax in islets.

Quantitative RT-PCR of wild-type islets cultured in control medium or medium containing 50 mM ribose, 33.3 mM glucose or 5 µM thapsigargin for 24 or 48 h. Relative RNA expression levels for (A) Puma (B) Bim and (C) Bax were calculated by normalising to the signal for β-actin in each sample and comparison to islets cultured in control medium. Mean ± SEM of 3-4 independent experiments is shown. Statistical significance *p<0.05, **p<0.01 compared with control (two-tailed paired t-test). (D) Western blotting was performed with antibodies to Bim, Puma and β-actin. Arrows indicate bands corresponding to Bim\(_{EL}\) and Bim\(_{L}\). NS is a non-specific band that serves as a loading control for the Bim immunoblot. Phosphorylation of Bim results in a shift of the Bim\(_{EL}\) and Bim\(_{L}\) bands to slightly greater molecular weight. Results are representative of 3 independent experiments.
Glucose-induced beta cell apoptosis

Figure 1

A i) Control, Glucose, Ribose

DNA fluorescence

Nuclei number

ii) % DNA fragmentation

Control L-Glucose L-Ribose D-Glucose D-Ribose

B i) Control, Glucose, Ribose

Cytochrome c

FSC

ii) % cytochrome c release

Control Glucose Ribose

C i) Control qVD.oph, Ribose, Ribose + qVD.oph

% DNA fragmentation

ii) % DNA fragmentation

Control qVD.oph Glucose Glucose + qVD.oph
Figure 2

A

% DNA fragmentation

Wild-type
IL-1R<sup>−/−</sup>
B<sup>B</sup>β<sup>−/−</sup>
Bid<sup>−/−</sup>

Control Glucose Ribose

B

Western blot analysis

Control Glucose IFNγ Glucose + IFNγ Blank IL-1β + IFNγ

iNOS β-actin

C

NO<sub>2</sub> production (pmol/10<sup>6</sup> cells)

Control Glucose IFNγ Glucose + IFNγ IL-1β + IFNγ

D

% DNA fragmentation

Control Glucose IFNγ Glucose + IFNγ IL-1β + IFNγ
Glucose-induced beta cell apoptosis

Figure 3

A

% cytochrome c release

Control | Ribose

B

% DNA fragmentation

Control | Ribose

C

% cytochrome c release

Control | Glucose

D

% DNA fragmentation

Control | Glucose
Figure 4

A

Bak\textsuperscript{-/}  
Bax\textsuperscript{-/}  
wild-type

Control  Ribose

% cytochrome c release

B

Control  Ribose

% DNA fragmentation

C

Control  Glucose

% cytochrome c release

D

Control  Glucose

% DNA fragmentation
Figure 6

A. puma

Fold change normalised to untreated islets

B. bim

Fold change normalised to untreated islets

C. bax

Fold change normalised to untreated islets

D. Western blot analysis

Control, Ribose, Glucose, Thepsigargin