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Assay for high glucose-mediated islet cell sensitization to apoptosis induced by streptozotocin and cytokines

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ABSTRACT

Pancreatic β-cell apoptosis is known to participate in the β-cell destruction process that occurs in diabetes. It has been described that high glucose level induces a hyperfunctional status which could provoke apoptosis. This phenomenon is known as glucotoxicity and has been proposed that it can play a role in type 1 diabetes mellitus pathogenesis. In this study we develop an experimental design to sensitize pancreatic islet cells by high glucose to streptozotocin (STZ) and proinflammatory cytokines [interleukin (IL)-1β, tumor necrosis factor (TNF)-α and interferon (IFN)-γ]-induced apoptosis. This method is appropriate for subsequent quantification of apoptotic islet cells stained with Tdt-mediated dUTP Nick-End Labeling (TUNEL) and protein expression assays by Western Blotting (WB).

INTRODUCTION

Type 1 diabetes mellitus results from autoimmune T-cell-mediated destruction of insulin-producing pancreatic islet β-cells (1). Although the mechanism of how this destruction takes place is not completely understood, β-cell apoptosis is known to participate in this process (2-4). IL-1β alone or in combination with other proinflammatory cytokines inhibits the glucose-induced insulin secretion and plays an important role in β-cell death by inducing toxic nitric oxide (NO) production and extracellular signal-regulated kinase (ERK) activation in the islet (5-8). Streptozotocin (STZ) has been used widely to produce animal models of diabetes. The effects of STZ on β-cells are similar to those of IL-1β and also seem to be mediated by NO (9) through alkylation, DNA damage and poly-ADP ribose polymerase (PARP) activation (10). Furthermore, there are mounting evidences that have recently shown an important role of β-cell death in type 2 diabetes mellitus (11).

The pancreatic microenvironment can play an important function in β-cell death, making these cells more susceptible or resistant to damage. High glucose concentration impairs islet function by disturbing glucose metabolism in β-cells mitochondria and induces apoptosis (12-14). In addition, it has been reported that high glucose concentration could enhance ß-cell vulnerability to toxic effects by increasing the expression of potential autoantigens on the cell membrane surface (15).

Based on the above-cited evidence, this study presents a method for the "in vitro" sensitization of islet cell, by using high subtoxic-to-toxic glucose concentrations, to STZ and cytokine-induced apoptosis. This method keeps the islets undamaged, so that, after trypsin dissociation, the cells can be stained by TUNEL. Also, the islets obtained were used to determine expression of apoptosis-related proteins like the cell death receptor Fas (CD95), Bcl-xL and Bcl-2, by WB. Fas seems to be implicated in β-cell apoptosis via an intracellular death domain (16) and proinflammatory cytokines can induce up-regulation of Fas expression on β-cells, making them susceptible to apoptosis in the presence of agonistic anti-Fas antibodies or interaction with Fas-ligand (FasL, CD95L)-expressing T-cells (17-19). The role of Fas in β-

cell apoptosis is still under debate and has been challenged by several studies (20, 21). In addition, up-regulation of several anti-apoptotic members of the Bcl-2 family of proteins, such as Bcl-2 and Bcl-xL has been closely associated with increased resistance to apoptosis and potentially to diabetes susceptibility (22-24). The expression of these genes may possibly be modified by the functional status of the β-cell, especially when associated with a high glucose concentration.

MATERIALS AND METHODS

Isolation and culture of rat islets

All animal procedures were performed with the approval of the Animal Ethical Use and Care Committee at the Cadiz University School of Medicine, Cadiz, Spain. Pancreatic islets were isolated from 200-250 g weight adult male Wistar rats, as described previously (25). In brief, animals were sacrificed and the pancreas was filled with a cold solution of colagenase P (1 mg/ml) in HBSS through a catheter introduced into the part of the choledoco running from the liver to the pancreas. Then the filled pancreas was extracted and digested at 37°C for 20 min. while being agitated. Islets were isolated from the digested pancreas by density gradients, collected and cultured in Petri plates (100 islets on a 60 cm plate with 5 ml of culture medium) with RPMI (Sigma, St Louis, MO) supplemented with 2 mM L-glutamine (Gibco,Invitrogen Limited, Paisley, UK), 10% fetal bovine serum (Gibco, Invitrogen Limited, Paisley, UK), 100 U/ml penicillin and 100 μg/ml streptomycin (Pen-Strep; Bio-Whittaker Europe, Verviers, Belgium) and containing the established glucose concentration for each experiment.

Sensitization protocol

First, the glucose concentration adequate for islet cell sensitization was determined by a dose-response experiment. This was performed using 2, 5.5, 11.1, 24.4 and 33.3 mM glucose and measuring different apoptosis rates by TUNEL-staining. The highest concentration which did not induce apoptosis "per se" was 24.4mM glucose; therefore this concentration was compared with 5.5 mM which was considered as the optimal concentration.

Sensitization to STZ-induced damage

Islets were cultured for a sensitization period (48h) with RPMI-completed medium supplemented with 5.5 or 24.4 mM glucose. After this period, the medium was replenished with fresh culture medium supplemented with 5.5. mM glucose for the all islets, maintaining the batches of islets separated according to the previous glucose concentration (during the sensitization period). Then STZ (Sigma, St Louis, MO) was added to the islet cultures for an incubation period of 24h.

Sensitization to cytokine-induced damage

Islets were cultured for an overnight period (≈14-16h) with RPMI-completed medium supplemented with 5.5 or 24.4 mM glucose. The culture medium was replenished with fresh medium maintaining the glucose concentration either with IL-1β (PeproTech EC Ltd, London, UK) alone or with IL-1β + TNF-α (PeproTech EC Ltd., London, UK) + IFN-γ (PeproTech EC Ltd, London, UK). These alternative mixtures of medium were added to the islets cultured for a incubation period of 24h. The specific cytokine (50 U/ml IL-1β; 1,000 U/ml IFN-γ; 1,000 U/ml TNF-α) and STZ (1.5 mM) concentrations used in these experiments were selected from previous studies (26-28).

Detection of apoptotic cells

Apoptotic cells were detected using the TUNEL technique. Following treatment with cytokines or STZ, islets were incubated for 15 min with Trypsin-EDTA, 0.25% trypsin, 1mM EDTA•4Na in Hanks´ balanced salt solution without Ca^{2+} and Mg^{2+}(Gibco, Invitrogen Limited, Paisley, UK) at 37°C, and the islet cells were gently dispersed. After washing with PBS, the cells were cytospun on Poly-L-lysine-coated slides, fixed in 4% methanol-free formaldehyde solution in PBS for 25 min at 4°C, and stored in 70% ethanol at -20°C until detection of apoptotic cells by TUNEL assay. The TUNEL assay was performed according to the manufacturer's instructions (Apoptosis Detection System, Fluorescein; Promega Corporation, Madison, WI) (29). The fluorescein-12dUTP-labeled DNA was directly visualized by fluorescence microscopy with excitation at 520 ± 20 nm, to allow counting of the proportion of apoptotic cells (nuclei with green fluorescence). Cell

nuclei were stained with propidium iodide (red fluorescence). Apoptotic and total nuclei were counted in a blinded fashion in two slides per condition and per experiment.

Western blot

Equal numbers of islets treated according to the various experimental conditions described above were lysed in 60 mM Tris-HCl pH-6-8, 2% SDS, 10% glycerol, 0.0012% bromophenol blue, and 5% β-mercaptoethanol. Islet lysates were boiled for 5 minutes and then loaded on a 10-12% SDS-polyacrylamide gel. An amount of lymphocytes of chronic lymphoid leukemia (CLL) equivalent to number of islet cells per condition were used as positive control for Bcl-2. Proteins were transferred to PDVF membrane and the blot was then incubated in blocking buffer (5% non-fat milk in 10 mM Tris-HCl, 1.15 M NaCl and 0.1% Tween-20) for 1h at room temperature. Next the blots were incubated with polyclonal antibodies against Fas (1:500 dilution; Santa Cruz Biotechnology Inc, Santa Cruz, CA), Bcl-xL (1:500 dilution; Santa Cruz Biotechnology Inc, Santa Cruz, CA), Bcl-2 (1:100 dilution; Abcam Ltd, Cambridge, UK), or actin (1/5000 dilution; Abcam Ltd, Cambridge, UK) for 2 h at room temperature, followed by incubation with the appropriate alkaline phosphatase-linked secondary antibody at room temperature for 1 h. Protein band detection was performed by adding 5-bromo-4-chloro-3-indolyl phosphate/ nitroblue tetrazolium (BCIP/NBT; Bio-Rad Laboratories, Hercules, CA) to the membranes. Data from the densitometry of the bands was captured using BioCaptMW-[BioCaptMW-1] software. In each experiment, band densities were normalized against actin, and the results are expressed as protein level to control islets (C = 1).

Statistical analysis

Results are presented as mean ± S.E.M. in at least three independent and separate experiments. Statistical analysis was performed using the Mann-Whitney test and a P value less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Effect of glucose concentration on islet cell apoptosis

It has recently been reported that chronic exposure to high glucose (for more than two days) leads to increased rat islet cell apoptosis (30). In the current study we first analyzed rat islet cell apoptosis in a glucose dose-response curve for 48 h; an increased percentage of islet cell apoptosis was observed with low (2 mM) and very high (33.3 mM) glucose concentrations in the medium, but not with 5.5, 11.1 or 24.4 mM glucose (Fig. 1).

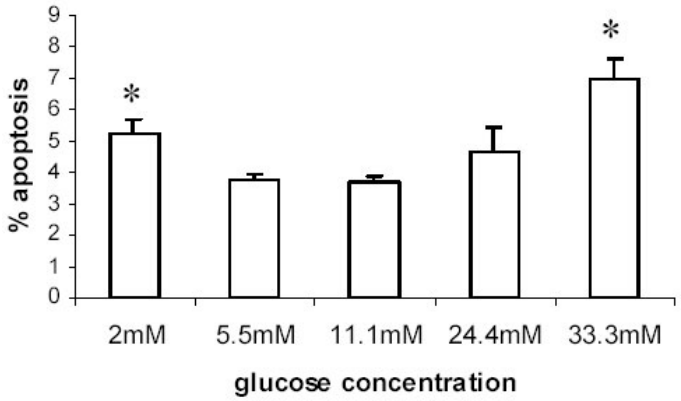

Fig. 1: Glucose dose–response curve. The islets were cultured with 2, 5.5, 11.1, 24.4 and 33.3 mM glucose for 48 h. Apoptotic cells were assessed by the TUNEL method under fluorescence microscopy. Data are expressed as the percentage of apoptotic cells. **P* < 0.05 vs control 5.5 mM and *P* < 0.05 vs control 11.1 mM.

Effect of sensitization period on STZ-induced islet cell apoptosis

Examination of TUNEL-stained cell preparations of islets maintained in 5.5 or 24.4 mM glucose for 48h and incubated with STZ for 24h was performed. We chose a dose of STZ that did not significantly increase rates of apoptosis in rat islet cells preincubated in 5.5mM glucose for 48h. Interestingly, apoptosis increased significantly (by 50%) when rat islets were incubated in 24.4mM glucose for 48 h with a low dose of STZ (1.5 mM) for an additional 24h period (Fig. 2). The rationale for STZ incubation with glucose 5.5 mM was to avoid competition between high glucose concentration and STZ for the glucose transporter GLUT-2 (31, 32).

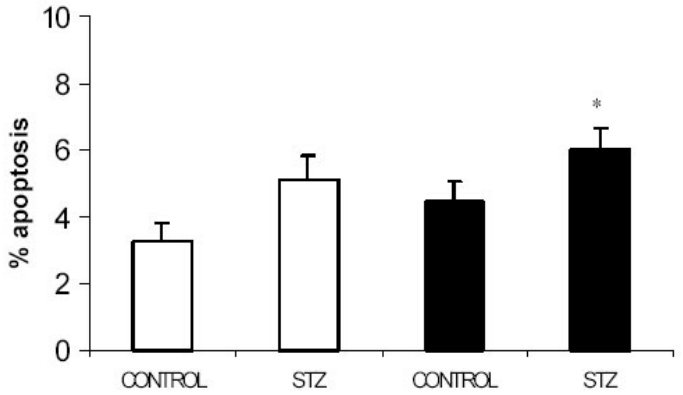

Fig. 2: Influence of different glucose concentrations (5.5 or 24.4 mM) on islet cell apoptosis induced by STZ (1.5 mM). The islets were cultured either with 5.5 or 24.4 mM glucose and exposed to STZ. Apoptotic cells were assessed by the TUNEL method under fluorescence microscopy. Data are expressed as the percentage of apoptotic cells. Open bars, islets cultured with 5.5 mM glucose; filled bars, islets cultured with 24.4 mM glucose. **P* < 0.05 vs control 24.4 mM glucose.

Effect of sensitization period on cytokine-induced islet cell apoptosis

We next analyzed whether high glucose also increases the sensitivity of rat islet cells to apoptosis induced by IL-1β+ TNF-α+ IFN-γ during the previous 24h. As shown in Fig. 3, a dose of cytokines that did not induce a significant increase in islet cell apoptosis at 5.5 mM glucose, was capable of inducing a significant two-fold increase in the apoptosis rates of islet cells maintained at 24.4 mM glucose. Rates of apoptosis in islet cells incubated in high glucose rose from 6.09 ± 0.78% to 13.07 ± 1.78% ($p < 0.01$), when cytokines were added. This result indicates that the sensitization period at 24.4 mM glucose makes cells more susceptible to the apoptosis effects induced by cytokines. The percentage of apoptotic cell in islets incubated with high glucose concentration (24.4 mM) and cytokines were significantly higher than in islets with cytokines but incubated in 5.5 mM glucose (13.07 ± 1.78% vs. 8.04 ± 1.56% ($p < 0.05$). Interestingly, this effect was only observed when the three cytokines were used in combination, since IL-1ß alone did not induce a significant increase in apoptosis at any glucose concentration tested. Collectively, these results suggest that although incubation for 48h with high glucose does not induce an increase in rat islet cell apoptosis, it potentiates the pro-apoptotic effects of cytokines and STZ.

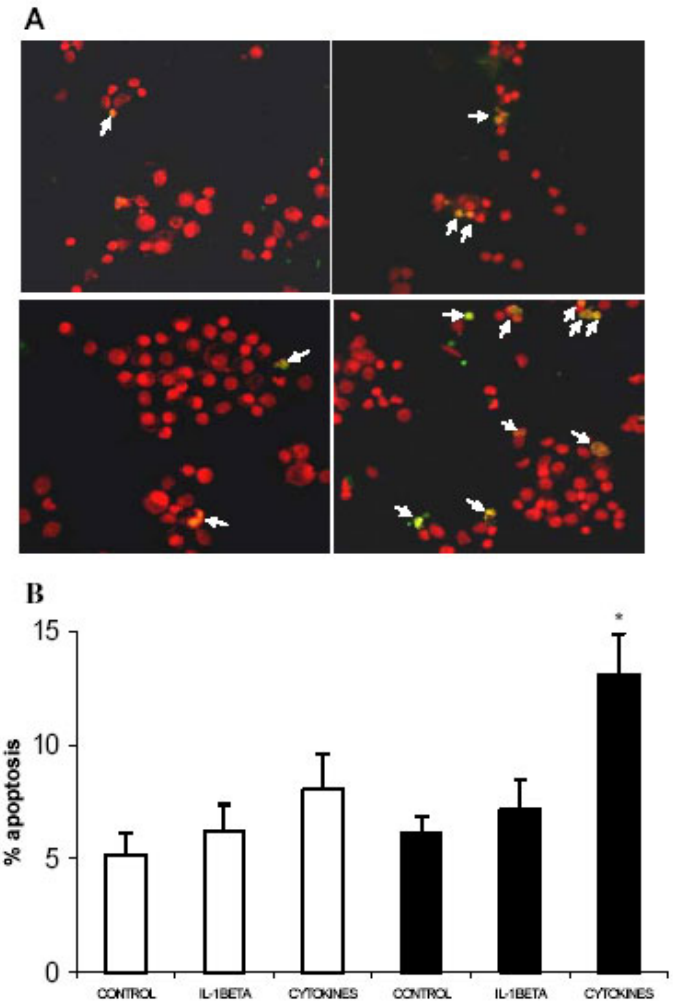

Fig. 3: Influence of different glucose concentrations (5.5 or 24.4 mM) on islet cell apoptosis induced by IL-1β and combined cytokines (IL-1β +IFN-γ +TNF-α). A. Representative microscopy images of apoptotic cells stained with TUNEL (green) and Propidium Iodide (red). 5.5 mM glucose control (upper left); 5.5 mM glucose with cytokines (upper right); 24.4mM glucose control (bottom left) and 24.4mM glucose exposed to cytokines (bottom right). Arrows indicate apoptotic cells (co-location of red fluorescence of all cells plus green fluorescence of apoptotic cells). **B.** Percentage of apoptotic cells cultured with 5.5 or 24.4 mM glucose and exposed to IL-1β and combined cytokines. Open bars, islets cultured with 5.5 mM glucose; filled bars, islets cultured with 24.4 mM glucose. **P* < 0.01 vs control 24.4 mM glucose and *P* < 0.05 vs. cytokines with 5.5 mM glucose.

Effect of sensitization period with high glucose on the expression of apoptosis-related proteins

Fas is a death receptor involved in apoptosis in various cell types including islet cells (16-19). To determine whether glucose concentration has any impact in the expression of Fas in rat islet cells treated either with cytokines or STZ, we performed WB analysis of islet extracts incubated with the cell-death inducers in normal (5.5 mM) and high (24.4mM) glucose concentrations. As it is shown in Figs. 4 and 5, incubation in high glucose for 48 h induced a significant increase ($p < 0.05$) in the

expression of Fas which was not modified when an incubation period with cytokines (Fig. 4) or STZ (Fig. 5) was performed. These results indicate that the sensitization period induces an increase in the expression of Fas in rat islet cells that is not associated with a rise in apoptosis. Moreover, high glucose potentiation of the apoptotic effects of cytokines and STZ does not correlate with an increase in Fas expression.

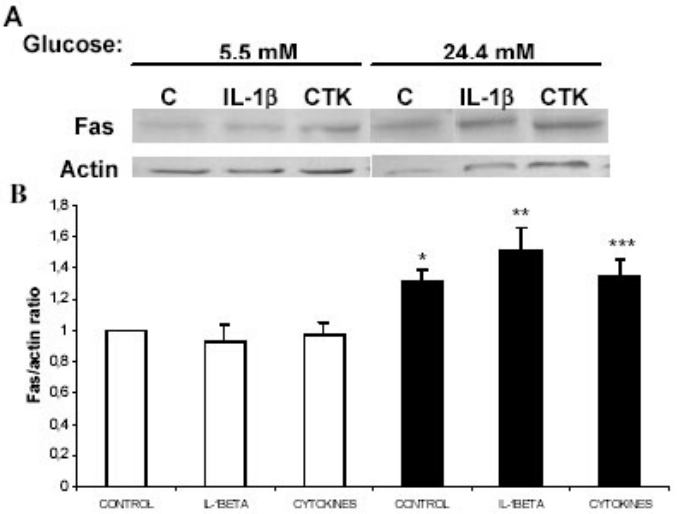

Fig. 4: Fas (CD95) expression in rat islets after exposure to IL-1β and combined cytokines (IL-1β, TNF-γ, IFN-α). (A) Immunoblotting of Fas. Islets were cultured overnight with 5.5 or 24.4 mM glucose and exposed to IL-1β alone or combined with TNF-α plus IFN-γ, for 24 h. Antibodies against Fas and actin were blotted in the same filter after stripping. One of at least three experiments is shown. Each experiment gave similar results. C, control; IL-1β, interleukin-1β; CTK, cytokines (IL-1β + TNF-α + IFN-γ). (B) Densitometric quantitation of Fas-to-actin ratio showed a significant difference between 5 mM and 24.4 mM in the three groups (control, IL-1 and CTK). Y-axis represents arbitrary units. Open bars, islets cultured with 5.5 mM glucose; filled bars, islets cultured with 24.4 mM glucose. **P*< 0.05 vs control, 5.5 mM glucose; ***P* < 0.05 vs. interleukin-1β, 5.5 mM glucose; *** *P* < 0.05 vs. cytokines, 5.5 mM glucose.

The anti-apoptotic proteins Bcl-xL and Bcl-2 expression were also studied after preculture with high glucose and treatment with the cytotoxic agents. As shown in Figures 6 and 7, we did not find any significant difference in the expression of either Bcl-xL or Bcl-2 in islets cultured at high or normal glucose concentration and treatment with cytokines (Fig. 6) or STZ (Fig. 7). However, while Bcl-xL was clearly detected, Bcl-2 was scarcely visible in all the conditions studied. As it has been described previously, basal expression of bcl-2 protein in pancreas islets seems to be very low and difficult to detect by WB (33, 34).

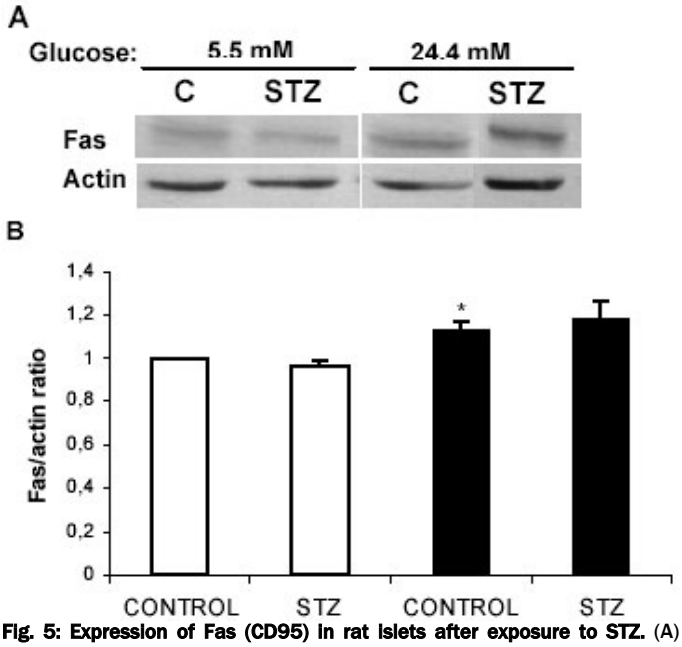

Fig. 5: Expression of Fas (CD95) in rat islets after exposure to STZ. (A) Immunoblotting of Fas. Islets were cultured for 48 h with 5.5 or 24.4 mM glucose and exposed to STZ for 24 h. Antibodies against Fas and actin were blotted in PVDF after stripping. One of at least three experiments is shown. Each experiment gave similar results. (B) Densitometric quantitation of Fas-to-actin ratio showed a significant difference between 5 mM and 24.4 mM. Y-axis represents arbitrary units. Open bars, islets cultured with 5.5 mM glucose; filled bars, islets cultured with 24.4 mM glucose. **P* < 0.05 vs control, 5.5 mM glucose.

However, the expression of other apoptosis-related genes could be analyzed after following the islet culture procedure shown in this work. Further studies will be required to clarify the molecular mechanisms responsible for the high-glucose enhancement of islet cell apoptosis induced by cytokines and STZ.

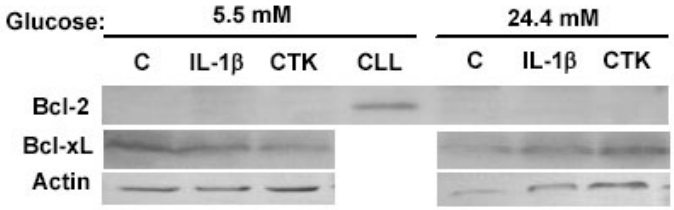

Fig. 6: Expression of Bcl-2 and Bcl-xL in rat islets after exposure to IL-1β and combined cytokines (IL-1β + TNF-γ + IFN-α). Islets were cultured overnight with 5.5 or 24.4 mM glucose and exposed to IL-1β alone or combined cytokines for 24 h. Immunoblottings were performed using antibodies against Bcl-2 and Bcl-xL. Actin levels were determined as loading control. Densitometric quantitation of both Bcl-2 and Bcl-xL-to-actin ratio did not generate significant differences (data not shown). One of at least three experiments is shown. Each experiment gave similar results. An amount of lymphocytes of chronic lymphoid leukemia (CLL) equivalent to number of islet cells per condition were used as positive control for Bcl-2. C, control; IL-1β, interleukin-1β; CTK, cytokines (IL-1β + TNF-α + IFN-γ).

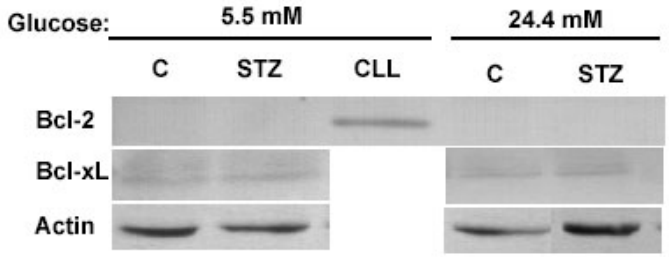

Fig. 7: Expression of Bcl-2 and Bcl-xL in rat islets after exposure to STZ. (A) Immunoblotting of Bcl-2, Bcl-xL and Fas. Islets were cultured for 48 h with 5.5 or 24.4 mM glucose and exposed to STZ for 24 h. The antibodies were blotted in different PVDF filters and antiactin after stripping was used for loading control. Densitometric quantitation of both Bcl-2 and Bcl-xL-to-actin ratio did not generate significant differences (data not shown). One of at least three experiments is shown. Each experiment gave similar results. An amount of lymphocytes of chronic lymphoid leukemia (CLL) equivalent to number of islet cells per condition were used as positive control for Bcl-2.

Conclusion

It is not well known whether high glucose concentration has any role in the apoptosis induced by STZ and cytokines in islet cells. We have designed an experimental method to assess the influence of glucose concentration on the effects of toxic agents classically involved in β-cell death and to analyze whether rat islet cell apoptosis induced by recognized islet cell toxic agents such as proinflammatory cytokines (IL-1β, TNF-α, IFN-γ) or STZ may be modulated by a pretreatment with high glucose concentration. In the study described here, we demonstrate that the presence of high glucose concentration in a preculture period enhances proinflammatory cytokine- and STZ-mediated apoptosis of rat pancreatic islet cells *in vitro*. Our results suggest that mechanisms involved in cytokine-induced apoptosis could be more amplified by the high glucose-induced hyperfunctional status of islet cells than the mechanisms implicated in STZ-mediated apoptosis. This effect could also be partially explained by recently reported glucose-induced IL-1β production by β-cells (35). Some of the differences between our study and others could be due to species, time course, glucose concentration in the culture media and experimental models used. Thus, although murine dispersed islet cells survive best at 11 mM glucose and apoptosis increases when glucose is increased or decreased (29), we show a similar U-shape curve but no differences between 5.5, 11 and 24.4 mM in whole rat islets. It is interesting to note that neither high glucose concentration nor the toxic agents used in this study caused marked apoptosis when used separately, and the higher levels of apoptosis were found when high glucose and cytokines, or STZ, existed simultaneously.

Our experimental design appears to be a feasible method for evaluating the influence of glucose on several stimuli causing β-cell damage and it seems to be adequate for determining synergistic and/or sensitization relationships between the effects of both high glucose concentration and potentially β-cells toxic agents (36).

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PROTOCOLS

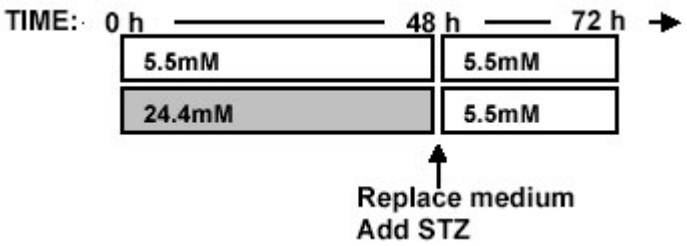
Rat islet isolation

1. Kill animal by an appropriate method.
2. Open the abdominal cavity.
3. Close choledoco exit to the intestine by clamping.
4. Introduce a catheter into the part of the choledoco running from the liver to the pancreas.
5. Connect the catheter to a syringe and inject 15-20 ml of a cold solution of colagenase P (1 mg/ml) in HBSS until the pancreas is full.
6. Extract the filled pancreas by cutting out around the edges.
7. Incubate for 17-20 min at 37°C in agitation.
8. Homogenize the preparation with a pasteur pipette and add 60 ml of HBSS+ BSA (1 mg/ml).
9. After 5-8 min placed in ice without movement, aspirate 15-20 ml from the surface to eliminate fat and other floating debris.
10. Pass through a strainer to eliminate non digested tissue and then into a 50 ml tube.
11. Centrifuge for 5 min, at 1000 rpm, 4°C and reject the supernatant.
12. Add to the "pellet" 10 ml. of Histopaque 1119 and resuspend. Put into 10 ml of Histopaque 1077 and 10 ml of HBSS+ BSA to form a density gradient.
13. Centrifuge for 20-25 min, at 2000 rpm, 4°C.
14. Take the interphase between Histopaque 1077 and HBSS+ BSA (10- 15 ml) and put it in a 50 ml tube.
15. Fill to 50 and wash by centrifuging for 5 min, at 100 rpm, 4°C.
16. Aspirate 30 ml of supernatant, homogenize the islet suspension gently with a pipette and put onto a black Petri plate.
17. Using the stereo microscope and micropipette, collect 100-150 islets on each 60 cm culture plate with 5 ml of culture medium).

Sensitization protocol

Sensitization to STZ-induced damage:

1. Culture islets for a sensitization period of 48h with RPMI-completed medium supplemented with 5.5 or 24.4 mM glucose.
2. Replace medium with fresh culture medium supplemented with 5.5 mM glucose for the all islets, maintaining separated the batches of islets according to the previous glucose concentration (during the sensitization period).
3. Add STZ to islet cultures.
4. Culture for 24h.



Sensitization to cytokine-induced damage:

1. Culture islets for an overnight period (≈14-16h) with RPMI-completed medium supplemented with 5.5 or 24.4 mM glucose.
2. Replace with fresh medium maintaining both glucose concentrations.
3. Add IL-1β (50 U/ml) alone or IL-1β + TNF-α (1000 U/ml) + IFN-γ (1000 U/ml).
4. Culture for an incubation period of 24h.