High glucose potentiates cytokine- and streptozotocin-induced apoptosis of rat islet cells: effect on apoptosis-related genes

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Abstract

Pancreatic β -cell apoptosis is known to participate in the β -cell destruction process that occurs in diabetes. A better understanding of how it takes place is essential for future development of therapeutic strategies aimed at preventing β -cell loss and diabetes. In this study we determine the possible role that high glucose concentration might play as an enhancer of cytokine- and streptozotocin (STZ)-mediated rat islet cell apoptosis *in vitro* and its relationship with potential changes in the expression of pro- and anti-apoptotic proteins. Rat islets treated with a cytokine combination (interleukin (IL)-1 β , tumor necrosis factor (TNF)- α and interferon (IFN)- γ) displayed a significant increase in islet cell apoptosis when the islets were incubated in 24.4 mM glucose compared with untreated islets at the same glucose concentration ($13.07 \pm 1.78\%$ vs $6.09 \pm 0.78\%$; $P < 0.01$) or islets incubated in 5.5 mM glucose concentration and cytokines ($13.07 \pm 1.78\%$ vs $8.04 \pm 1.56\%$; $P < 0.05$). IL-1 β alone did not induce a

significant increase in the apoptotic rates in islet cells cultured at normal or high glucose concentrations. STZ significantly increased islet cell apoptosis when islets were cultured in 24.4 mM glucose concentration compared with untreated islets at the same glucose concentration ($6.02 \pm 0.62\%$ vs $4.44 \pm 0.63\%$; $P < 0.05$). High glucose induced an increase in Fas expression in the islet cells, and this increase was maintained after cytokine or STZ treatment. However, the expression of anti-apoptotic mediators such as bcl-2 and bcl-xL did not show any significant change. These results suggest that cytokine- and STZ-mediated apoptotic effects on islet cells might be mediated by a glucose-induced hyperfunctional status and associated with an increase in Fas (Apo-1, CD-95) expression and no changes in the expression of the anti-apoptotic proteins bcl-xL and bcl-2.

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Introduction

Type 1 diabetes mellitus results from autoimmune T-cell-mediated destruction of insulin-producing pancreatic islet β -cells (Castaño & Eisenbarth 1990). Although the mechanism of this destruction is not completely understood, β -cell apoptosis is known to participate in this process (O'Brien *et al.* 1997, Kurrer *et al.* 1997, Augstein *et al.* 1998). Interleukin (IL)-1 β alone or in combination with other pro-inflammatory cytokines inhibits the glucose-induced insulin secretion and plays an important role in β -cell death by inducing toxic nitric oxide (NO) in the islet (Kaneto *et al.* 1995, Dunger *et al.* 1996, Mandrup-Poulsen 1996). Streptozotocin (STZ) has been used widely to produce animal models of diabetes. Although the effect of STZ and IL-1 β on β -cells seems to be mediated by NO (Turk *et al.* 1993), it is generally accepted that the mechanism for STZ toxicity is via alkylation, DNA damage and poly-ADP ribose polymerase (PARP) activation (Masutani *et al.* 1999) and IL-1 β via extracellular signal-regulated kinase (ERK) (Pavlovic *et al.* 2000).

High glucose concentration impairs islet function by disturbing glucose metabolism in the mitochondria of β -cells and could induce apoptosis (Sandler *et al.* 1990, Laybutt *et al.* 2001, Maedler *et al.* 2001). In addition, it has been reported that high glucose could increase β -cell vulnerability to toxic damage by increasing the expression of potential autoantigens on the cell membrane surface (Aguilar-Diosdado *et al.* 1994).

Fas has also been postulated to play a role in the autoimmune β -cell damage. The cell death receptor, Fas (CD95), seems to be implicated in β -cell apoptosis via an intracellular death domain (Krammer 2000). Cytokines can induce up-regulation of Fas expression in β -cells, making them susceptible to apoptosis in the presence of agonistic anti-Fas antibodies, or interaction with Fas-ligand (FasL, CD95 L)-expressing T-cells (Stassi *et al.* 1995, Yamada *et al.* 1996, Loweth *et al.* 1998). The role of Fas in β -cell apoptosis is still the subject of debate, and has been challenged by several studies (Allison & Strasser 1998, Thomas *et al.* 1999). 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 strongly associated with increased resistance to apoptosis and potentially linked with diabetes susceptibility (Garchon *et al.* 1994, Lamhamedi-Cherradi *et al.* 1998, Hanke 2000).

The aim of the current study was to determine whether high glucose concentration modifies the apoptosis mediated by STZ, IL-1 β or a combination of proinflammatory cytokines such as tumor necrosis factor (TNF)- α , interferon (IFN)- γ and IL-1 β in rat pancreatic islet cells. In addition, we explored the expression level of potentially related apoptotic and anti-apoptotic molecules in rat islet cells treated with these various cell death inducers and different glucose concentrations. Our results suggest that high glucose concentration (a) potentiates cytokine- and STZ-mediated rat islet cell apoptosis and (b) increases the expression of Fas in these cells.

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 adult male Wistar rats, as described previously (McDaniel *et al.* 1983). Isolated islets were cultured in RPMI medium (Sigma) supplemented with 2 mM L-glutamine (Gibco), 10% fetal bovine serum (Gibco), 100 U/ml penicillin and 100 μ g/ml streptomycin (Pen-Strep; Bio-Whittaker Europe, Verviers, Belgium), and containing either 5.5 or 24.4 mmol/l glucose (Ling *et al.* 1994). A dose-response experiment was performed using 2, 5.5, 11.1, 24.4 and 33.3 mM glucose for measurement of different apoptosis rates.

Cytokines and STZ treatment

To determine whether glucose has any influence on cytokine-mediated apoptosis in rat islet cells, isolated islets cultured for 16–20 h with RPMI containing 5.5 or 24.4 mM glucose were then exposed for 24 h to either recombinant human IL-1 β (PeproTech EC Ltd, London, UK) alone or combined with recombinant human IFN- γ (PeproTech EC Ltd), and recombinant human TNF- α (PeproTech EC Ltd). Cytokine concentrations used in these experiments (50 U/ml IL-1 β , 1000 U/ml IFN- γ , 1000 U/ml TNF- α) were selected from previous studies (Eizirik *et al.* 1994, Delaney *et al.* 1997, Hoorens & Pipeleers 1999).

To analyze the effect of different glucose concentrations on STZ-induced apoptosis in rat islet cells, a different set of islets was cultured for 48 h with RPMI containing either 5.5 or 24.4 mM glucose. After that period of time,

islets were exposed to STZ (1.5 mM; Sigma) for an additional 24 h period.

Detection of apoptotic cells

Apoptotic cells were detected using the TUNEL (Tdt-mediated dUTP nick-end labeling) technique. Following cytokines or STZ treatment, islets were incubated for 15 min with trypsin-EDTA: 0.25% trypsin, 1 mM EDTA·4Na in Hanks' balanced salt solution without Ca²⁺ and Mg²⁺ (Gibco) at 37 °C, and islet cells were gently dispersed. After washing with PBS, 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) (Efanova *et al.* 1998). The fluorescein-12-dUTP-labeled DNA was directly visualized by fluorescence microscopy with excitation at 520 $\pm$ 20 nm, to allow counting of the percentage 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, of more than 1000 cells with two slides per condition and per experiment.

Western blot

Equivalent numbers of islets treated with the various experimental conditions mentioned 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 min and then loaded on a 10–12% SDS-polyacrylamide gel. Proteins were transferred to polyvinylidene fluoride (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 1 h at room temperature. Next, blots were incubated with polyclonal antibodies against Fas (1:500 dilution; Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA), bcl-xL (1:500 dilution; Santa Cruz Biotechnology), bcl-2 (1:100 dilution; Abcam Ltd, Cambridge, UK) or actin (1/5000 dilution; Abcam Ltd) 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/ nitro-blue tetrazolium (BCIP/NBT; Bio-Rad) to the membranes. Densitometry of the bands was quantitated using BioCaptMW-1 software.

Statistical analysis

Results are presented as means $\pm$ S.E.M. in at least three independent and separate experiments. Statistical analysis

Figure 1 Detection of apoptotic rat islet cells by fluorescence microscopy (TUNEL method). Islets were cultured for 16–20 h with 5.5 or 24.4 mM glucose and exposed to IL-1 β +TNF- α +IFN- γ for an additional period of 24 h. At the end of treatment, islets were incubated with trypsin to obtain dispersed cells. (A) Control 5.5 mM glucose; (B) control 24.4 mM glucose; (C) cytokines with 5.5 mM glucose; (D) cytokines with 24.4 mM glucose. Arrows indicate apoptotic cells (co-location of red propidium iodide fluorescence of all cells plus green fluorescence of apoptotic cells by fluorescein).

was performed using the Mann–Whitney test and a *P* value less than 0.05 was considered statistically significant.

Results

Effect of high glucose on islet cell apoptosis

It has been recently reported that chronic exposure to high glucose leads to increased rat islet cell apoptosis (Piro *et al.* 2002). In that study, the apoptotic effect required at least 3 days of culture in high glucose concentration. In the current study we first analyzed rat islet cell apoptosis after incubation with 5.5 and 24.4 mM glucose for 2 days. As shown in Fig. 1A and B, similar numbers of apoptotic cells per field were found in rat islet preparations incubated with either 5.5 or 24.4 mM glucose and, following quantitation, no significant differences in the apoptotic rates in these cells were found (Figs 2, 3 and 4). Based on these results, we decided to examine whether incubation with high glucose (24.4 mM) concentrations for 2 days might increase the sensitivity of islet cells to the apoptotic effects induced by cytokines or STZ. To ensure we had chosen the correct glucose concentration as control for the apoptotic rate of rat islet cells, a dose–response curve was produced. We observed an enhanced percentage of islet cell apoptosis 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. 2).

Effect of high glucose on cytokine-induced islet cell apoptosis

Examination of TUNEL-stained cell preparations of islets maintained in 5.5 or 24.4 mM glucose for 48 h, and incubated with IL-1 β +TNF- α +IFN- γ for the last 24 h,

Figure 2 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.

Figure 3 Influence of different glucose concentrations (5.5 or 24.4 mM) on islet cell apoptosis induced by IL-1 β and combined cytokines (IL-1 β +IFN- γ +TNF- α). The islets were cultured either with 5.5 or 24.4 mM glucose and exposed to IL-1 β and combined cytokines (IL-1 β +IFN- γ +TNF- α). 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 <0.01 vs control 24.4 mM glucose and P <0.05 vs cytokines with 5.5 mM glucose.

suggested that the number of apoptotic cells was increased when the islets were incubated in high glucose concentrations compared with normal glucose levels (Fig. 1C and D). To confirm whether the apoptotic rates were increased, we performed a blinded systematic quantitation of the apoptotic rates in islet cells treated with the aforementioned conditions. As shown in Fig. 3, a dose of cytokines that was not able to induce a significant increase in islet cell apoptosis at 5.5 mM glucose was capable of inducing a significant two-fold increase in the apoptotic rates of islet cells maintained in 24.4 mM glucose. Apoptotic rates in islet cells incubated in high glucose increased from 6.09 ± 0.78 to $13.07 \pm 1.78\%$ (P <0.01) when cytokines were added. This result indicates that although incubation with high glucose (24.4 mM) for 2 days does not increase rat islet cell death rates it makes cells more susceptible to the apoptotic effects induced by cytokines. The apoptotic cell rates in islets incubated in high glucose (24.4 mM) and treated with cytokines were significantly higher than the rates in islets treated with cytokines but incubated in 5.5 mM glucose (13.07 ± 1.78 vs $8.04 \pm 1.56\%$, P <0.05, Fig. 3). Interestingly, this effect was only observed when the three cytokines were used in combination since IL-1 β alone did not induce a significant increase in apoptosis at any glucose concentration tested.

Effect of high glucose on STZ-induced islet cell apoptosis

We next analyzed whether high glucose also increases the sensitivity of rat islet cells to apoptosis induced by STZ. As shown in Fig. 4 we chose a dose of STZ that did not significantly increase apoptotic rates in rat islet cells incubated in 5.5 mM glucose for 48 h. Interestingly, apoptosis was significantly increased (50%) when rat islets

Figure 4 Influence of different glucose concentrations (5.5 or 24.4 mM) on islet cell apoptosis induced by STZ. 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.

were incubated in 24.4 mM glucose for 48 h and with a low dose of STZ for an additional 24 h period (Fig. 4).

Collectively, the results described so far indicate that although incubation for 48 h with high glucose does not induce an increase in rat islet cell apoptosis, it potentiates the pro-apoptotic effects of cytokines and STZ in these cells.

Effect of high glucose on the expression levels of Fas, bcl-2 and bcl-x after cytokine or STZ treatment

Fas is a death receptor involved in apoptosis induced in various cell types including islet cells (Krammer 2000, Yamada *et al.* 1996, Stassi *et al.* 1995, Loweth *et al.* 1998). To determine whether glucose has any impact on the expression levels of Fas in rat islet cells treated with cytokines or STZ, we performed Western blot analysis of islet extracts incubated with the cell-death inducers in normal or high glucose. First, incubation of islet cells in high glucose for 2 days induced a significant increase (P <0.05) in the expression of Fas (Figs 5 and 6). Interestingly, cytokines treatment of islets incubated in either 5.5 or 24.4 mM glucose did not induce a significant increase in the expression levels of Fas in these cells compared with control islets (Fig. 5). Similarly, STZ treatment did not significantly change Fas expression in rat islets incubated with 5.5 or 24.4 mM glucose compared with control islets incubated in the same glucose concentration (Fig. 6).

Taken together, these results indicate that high glucose induces an increase in the expression of Fas in rat islet cells and this enhancement does not correlate with a rise in the apoptotic rates. Moreover, high glucose potentiation of the apoptotic effects of cytokines and STZ in rat islet cells does not correlate with a further increase in Fas expression levels in these cells.

Figure 5 Expression of bcl-2, bcl-xL and Fas (CD-95) in rat islets after exposure to IL-1 β and combined cytokines (IL-1 β , TNF- α , IFN- γ). (A) Immunoblotting of bcl-2, bcl-xL and Fas. Islets were cultured overnight with 5.5 or 24.4 mM glucose and exposed to IL-1 β alone or to IL-1 β plus TNF- α plus IFN- γ , for 24 h. The antibodies were blotted in different PVDF filters and antiactin in the same filter of antiFas after stripping. One of at least three experiments is shown. Each experiment gave similar results. Lymphocytes of chronic lymphoid leukemia (CLL) were used as positive control for bcl-2. C, control; IL-1 β , interleukin-1 β ; CTK, cytokines (IL-1 β +TNF- α +IFN- γ). (B) Densitometric quantitation of Fas-to-actin ratio. 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-1 β , 5.5 mM glucose; *** P <0.05 vs cytokines, 5.5 mM glucose.

The expression levels of bcl-xL and bcl-2 anti-apoptotic proteins were also studied in rat islet cells incubated in different glucose concentrations and treated with the cytotoxic agents. As shown in Figs 5 and 6, we did not find any significant difference in the expression levels

of both bcl-xL and bcl-2 in rat islets cultured at high or normal glucose, and treated with STZ or cytokines (Figs 5 and 6). However, while bcl-xL was clearly detected, bcl-2 was scarcely visible in all the conditions studied (Figs 5 and 6).

Figure 6 Expression of bcl-2, bcl-xL and Fas (CD-95) 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 in the same filter of antiFas after stripping. One of at least three experiments is shown. Each experiment gave similar results. Lymphocytes of chronic lymphoid leukemia (CLL) were used as positive control for bcl-2. (B) Densitometric quantitation of Fas-to-actin ratio. 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.

Discussion

It has been shown that high glucose concentration increases the expression of autoantigens on the β -cell membrane surface (Aguilar-Diosdado *et al.* 1994). Importantly, intensive insulin therapy and tight control of blood glucose at the onset of type 1 diabetes results in an improvement in beta-cell function (Shah *et al.* 1989). This improvement seems to be caused by insulin-induced β -cell rest. Furthermore, insulin treatment has been shown to prevent type 1 diabetes in murine (Gottfredsen *et al.* 1985) and human subjects at high risk of developing the disease (Keller *et al.* 1993). Moreover, *in vitro* studies have shown that chronic exposure of human or rat islets to high glucose increases the apoptotic rates in these islet cells (Maedler *et al.* 2001). However, whether high glucose concentration has any role on the apoptosis induced by cytokines in islet cells is not known. In the current study, we analyzed whether rat islet cell apoptosis induced by recognized islet cell toxic agents such as proinflammatory cytokines (IL-1 β , TNF- α , IFN- γ) or STZ is modulated by glucose concentration. In the study described herein, we demonstrate that the presence of high glucose concentration enhances proinflammatory cytokine- and STZ-mediated apoptosis of rat pancreatic islet cells *in vitro*.

In these experiments, we incubated rat islets in a high glucose concentration for a period of time (48 h) that does not result in islet cell apoptosis, as previously reported (Piro *et al.* 2002). In addition, we chose an incubation period (24 h) and concentrations of STZ and cytokines that did not induce islet cell apoptosis when incubated in normal glucose, as previously reported (Eizirik *et al.* 1997, Hoorens & Pipeleers 1999, Liu *et al.* 2002, Thomas *et al.* 2002). Importantly, under these conditions of high glucose, the apoptotic effects of cytokines and STZ in rat islet cells were strikingly potentiated. However, high glucose potentiation of the apoptotic effects of cytokines was greater than the potentiation of the apoptotic effects of STZ in these conditions. Both STZ (Turk *et al.* 1993) and cytokines (Kaneto *et al.* 1995, Dunger *et al.* 1996) have been shown to induce apoptosis in β -cells and it is known that glucose concentration modifies mouse islet loss after STZ treatment (Eizirik *et al.* 1988), but the mechanisms involved are not well understood (Suarez-Pinzon *et al.* 1994). Our results suggest that mechanisms involved in cytokine-induced apoptosis could be more amplified by high glucose-induced hyperfunctional status of islet cells than the mechanisms implicated in STZ-mediated apoptotic effects. This effect also could be partially explained by the recently reported glucose-induced IL-1 β production by β -cells (Maedler *et al.* 2002). Some 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 enhances when glucose is increased or decreased

(Efanova *et al.* 1998), we show a similar U-shape curve but with no differences between 5.5, 11 and 24.4 mM in whole rat islets.

The molecular mechanisms of islet cell apoptosis are unclear. Increased expression of Fas has been related to β -cell damage. The mechanisms underlying glucose-induced β -cell death in human islets involve the up-regulation of Fas receptors, which can interact with the constitutively expressed FasL in neighboring β -cells (Loweth *et al.* 1998). Fas-FasL interaction leads to cleavage of procaspase-8 to caspase-8 and activated caspase-8 promotes caspase-3 activation and DNA fragmentation (Stennicke & Salvesen 2000, Maedler *et al.* 2001). Human islets constitutively express FasL (Loweth *et al.* 1998) whereas islets from 2- to 3-month-old rats – the age of the rats chosen for our experiments – do not express FasL (Hanke 2000). Similar to the studies with human islets, we found that incubation with high glucose concentration for 2 days induces an increase in the expression of Fas in rat islet cells. This increase was sustained but not amplified when islets were treated with STZ or cytokines. Collectively, these results suggest that increased expression of Fas receptor in rat islets is not a predictor of enhanced islet cell apoptosis and other mechanisms might be implicated in the pro-apoptotic effect of STZ and cytokines in rat islet cells incubated in high glucose concentrations. Based on this, we analyzed whether the expression levels of two potential anti-apoptotic intracellular mediators – such as bcl-2 and bcl-xL that have been associated with increasing resistance to apoptosis (Garchon *et al.* 1994, Lamhamedi-Cherradi *et al.* 1998, Hanke 2000) – were down-regulated after treatment with STZ or cytokines in high glucose. The results indicate that bcl-2 was scarcely expressed and bcl-xL, although detected, did not show any significant variation in the expression level under any of the conditions studied. These findings suggest that bcl-2 and bcl-xL do not play an important role in STZ- and cytokine-induced apoptosis in rat islet cells incubated in high glucose concentrations. 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.

In conclusion, our studies demonstrate that: (a) STZ- and cytokine-induced apoptosis of rat islet cells is potentiated by high glucose concentration in the culture medium; and (b) high glucose is associated with increased Fas expression in rat islet cells. As diabetes mellitus is caused by the loss of β -cell mass, mainly caused by apoptosis, preventive interventions should be focused on achieving strict glucose control to avoid hyperfunctional status of pancreatic islet β -cells.

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