Effect of prolonged *in vitro* exposure to high glucose on neonatal porcine pancreatic islets

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Abstract

Prolonged exposure to high glucose can influence the function, growth, and survival of pancreatic β -cells. In this study, we examine the effects of prolonged *in vitro* exposure to high glucose on neonatal porcine β -cells, a potentially useful source of insulin-producing cells for clinical islet transplantation. Neonatal porcine islets were prepared by culturing collagenase-digested pancreases for 1 week in 5.6 mM glucose, followed by an additional week in either 5.6, 10.0, or 28.0 mM glucose. An additional 2 days of culture in 5.6 mM glucose followed for recovery from high glucose. The 7-day culture period in 28.0 mM glucose failed to irreversibly impair glucose responsiveness and also caused a modest increase in β -cell mass. Immunostaining revealed that

precursor cell differentiation was responsible for the increase in β -cell mass rather than β -cell proliferation. Islet cell survival was also assessed by a DNA fragmentation assay (TUNEL stain) to determine β -cell susceptibility to apoptosis after exposure to high glucose. Interestingly, although the total number of apoptotic islet cells did not drastically change after a week of culture in either 5.6, 10.0, or 28.0 mM glucose (25% TUNEL-positive), neither did the percentage of apoptotic β -cells. These encouraging results further support the use of neonatal porcine islets for clinical transplantation because of their ability to resist the cytotoxic effects of high glucose on islet function and survival.

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Introduction

Pancreatic β -cells not only depend on glucose as a source of fuel, but also require it as a growth factor as well as for the regulation of glucokinase and insulin gene transcription (Liang *et al.* 1992, Chen *et al.* 1994, Gasa *et al.* 2000). Elevated glucose concentrations can have beneficial effects, such as stimulating β -cell growth, but also detrimental effects, such as causing impaired insulin secretion and cell death (Svenne *et al.* 1980, Kim *et al.* 2005). The adult human β -cell has been shown to be particularly susceptible to high glucose resulting in reduced glucose-stimulated insulin secretion, disproportionately elevated proinsulin secretion, and reduced islet insulin content (Eizirik *et al.* 1992, Ling & Pipeleers 1996, Hostens *et al.* 1999, Marshak *et al.* 1999). In addition to impaired β -cell function, a recent study demonstrated that high glucose can cause increased β -cell death by tilting the balance of proapoptotic and anti-apoptotic Bcl proteins towards apoptosis (Federici *et al.* 2001). In addition, exposure to high glucose can impair glucose signaling in β -cells by decreasing GLUT2 and glucokinase gene expression (Tsuboi *et al.* 2006). However, unlike adult β -cells, fetal β -cells appear less susceptible to the detrimental effects of chronic exposure to high glucose (Tuch & Zheng 1993). Immature fetal β -cells respond poorly to

glucose (Korsgren *et al.* 1991), whereas after birth, β -cells mature becoming glucose-responsive (Kuhl *et al.* 1980). Islets from neonatal animals like pigs provide an interesting model to study the effects of high glucose exposure on β -cell function and survival as well as differentiation from precursors. Neonatal porcine islets contain differentiated endocrine cells as well as their precursors which are identified by cytokeratin-7 (CK7) immunoreactivity (Trivedi *et al.* 2001, Rayat *et al.* 2003).

These islets can reverse chemically induced hyperglycemia in diabetic rodents and large animal models (Korbitt *et al.* 1996, Cardona *et al.* 2006, Kin *et al.* 2005). This *in vivo* transplantation model has been shown to be effective for recapitulating the growth and differentiation of islet cells (Lukinius & Korsgren 2001). In the time required for this to occur, neonatal porcine islet grafts contain a suboptimal β -cell mass, which is insufficient to reverse hyperglycemia until 6–8 weeks posttransplant. During this time, the neonatal porcine islet graft is exposed to prolonged hyperglycemia, which may affect islet function, growth, and survival. The effects of high glucose exposure have not yet been studied to date, therefore in the present study neonatal porcine islets were exposed to slightly elevated (10.0 mM) and high (28.0 mM) glucose concentrations and assessed for β -cell function, growth, and survival in culture.

Materials and Methods

Neonatal porcine islet isolation and culture

Islets were isolated from Landrace–Yorkshire neonatal pigs (Swine Research and Technology Center, University of Alberta) aged 1–2 days old (1.5–2.0 kg body weight) of either sex as previously described by Korbitt *et al.* (1996). Briefly, the pancreases were removed, cut into small pieces, and digested with 1.0 mg/ml collagenase XI (Sigma). After filtration through a nylon screen (500 μ m), islets were cultured for 7 days in Hams F10 medium (Gibco) containing 5.6 mM glucose supplemented with 50 μ M isobutylmethylxanthine (IBMX; ICN Biomedicals, Montreal, Ontario, Canada), 0.5% BSA (Sigma), 2 mM L-glutamine, 10 mM nicotinamide (BDH Biochemical, Poole, Dorset, UK), 100 U/ml penicillin, and 100 μ g/ml streptomycin (Cambrex Bio Science, East Rutherford, New Jersey, USA) at 37 °C in humidified 5% CO₂–95% air with a change in the medium every 2 days. Following the initial week of culture in 5.6 mM glucose, islets were divided into three groups and cultured in supplemented Hams F10 medium containing either 5.6, 10.0, or 28.0 mM glucose for an additional 7 days with a change in the medium every 2 days. Islets from each group were then returned to 5.6 mM glucose for an additional 2 days.

Cellular insulin and DNA content analysis

Determination of cellular insulin content was accomplished by measuring duplicate samples by RIA (Diagnostic Products Corp., Los Angeles, CA, USA). Samples were sonicated in 2 mM acetic acid containing 0.25% BSA, centrifuged (800 g, 15 min), and the supernatants were collected and stored at –20 °C until the time of assay. The DNA content was analyzed using PicoGreen, an ultra-sensitive fluorescent nucleic acid stain for double-stranded DNA (Molecular Probes, Eugene, OR, USA), according to the manufacturer's instructions. Duplicate aliquots were washed in citrate buffer (150 mM NaCl, 15 mM citrate, 3 mM EDTA, pH 7.4) and stored as cell pellets at –20 °C. Cell pellets were resuspended in lysis buffer (10 mM Tris, 1 mM EDTA, 0.5% Triton X-100, 4 °C, pH 7.5), sonicated, and then analyzed (Korbitt *et al.* 1996).

Glucose-stimulated insulin secretion

Islet insulin secretory activity in response to an *in vitro* stimulatory glucose challenge was assessed using a static incubation assay (Korbitt *et al.* 1996). Islets were washed twice with Hams F10 medium containing 0.5% BSA and 2.8 mM glucose, and samples were taken for determination of cellular insulin content. Islets were cultured in 24-well plates and incubated in 1.5 ml of Hams F10 medium supplemented with 0.5% BSA and either 2.8 or 20.0 mM glucose for 120 min. At the end of the incubation, supernatants were collected for measurement of insulin release by RIA. Insulin secretion was calculated by dividing the insulin released into

the supernatant by the cellular insulin content of the islets (percent of content). Stimulation indices were calculated by dividing the percentage of insulin released at 20.0 mM glucose by that released at 2.8 mM glucose. Insulin release per β -cell was calculated by dividing the amount of insulin released into the culture medium by the number of β -cells. Based on the determination of total islet cellular DNA content and the percentage of insulin-positive cells, the following equation was used to calculate the total number of β -cells (Korbitt *et al.* 1996):

$$\frac{\text{Total DNA content}}{7.1 \text{ pg DNA/cell}} \times \frac{\% \text{ Insulin-positive cells}}{100} = \text{number of } \beta\text{-cells}$$

Differentiation, proliferation, and apoptosis

To determine the percentage of insulin-positive β -cells, CK7-positive ductal epithelial cells, and proliferating cells, islets were dissociated into single cell suspensions to facilitate quantification of stained cells (Korbitt *et al.* 1996). Islets were dissociated by mechanical disruption using siliconized glass pipettes at 37 °C in calcium-free HBSS medium supplemented with 1 mM EGTA and 0.5% BSA for 7 min before addition of trypsin (25 μ g/ml; Boehringer Mannheim, Laval, Canada) and DNase (4 μ g/ml, Boehringer), and further pipetting for 10 min. Cells were washed, resuspended in PBS, and allowed to adhere onto Histobond microscope slides (Paul Marienfeld GmbH & Co. KG, Lauda-Koenigshofen, Germany) before fixation in Bouin's fixative for 12 min. Microwave antigen retrieval for CK7 staining was performed for 1.5 min in 50 ml of target retrieval solution, pH 9 (S2368, Dako, Mississauga, Canada). Blocking was performed with 20% normal goat serum (Fischer Scientific, Edmonton, AB, Canada) for 30 min. Primary antibodies were purchased from Dako and used at the following concentrations: 1:1000 guinea pig anti-porcine insulin, 1:25 mouse anti-human CK7, and 1:100 mouse anti-rat proliferating cell nuclear antigen (PCNA) for 1 h at room temperature in a humidified chamber followed by two washes in PBS before addition of the secondary antibody. Secondary antibodies were as follows: fluorescein isothiocyanate (FITC)-conjugated goat anti-guinea pig and Cy3-conjugated donkey anti-mouse, both purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA) and used at a dilution of 1:200 for 1 h. Double staining for insulin and PCNA was performed in sequential order of primary followed by secondary antibodies. The percentage of PCNA-positive islet cells was calculated by dividing the number of PCNA-positive cells by the total number of cells. The percentage of proliferating β -cells was determined by dividing the number of PCNA and insulin double positive cells by the total number of insulin-positive cells. Apoptotic cells were detected by TUNEL staining (Promega) according to the manufacturer's instructions on intact islets fixed in 4% paraformaldehyde for 30 min, then

embedded in a 2% low-melting point agarose solution and allowed to harden at 4 °C before processing, paraffin embedding, and sectioning (5 μ m). Sections of islets were stained for apoptotic cells and counterstained for insulin. Slides were coverslipped with Vectashield (Vector) and viewed using a Zeiss Axioskop II fluorescent microscope with a Coolsnap camera. The percentage of TUNEL-positive cells was determined by dividing the number of TUNEL-positive cells by the total number of cells. The percentage of apoptotic β -cells was determined by dividing the number of TUNEL and insulin double positive cells by the total number of insulin-positive cells. Cell counts were performed on a minimum of 500 cells per sample and percentages were calculated as the number of positive cells per 500.

Statistical analysis

Results are presented as mean $\pm$ S.E.M. of three independent experiments performed with islets prepared from at least three pancreases. Statistical comparisons were performed using the one-way ANOVA test with SPSS statistical software, version 12.0 for Windows (Chicago, IL, USA). A *P* value of <0.05 was considered significant.

Results

Effect of high glucose on neonatal porcine islet recovery and insulin secretory activity

Immediately after collagenase digestion of neonatal porcine pancreases, digested tissue was cultured in Hams F10 medium containing 5.6 mM glucose for 1 week. The resultant islets were then switched to media containing either 5.6, 10.0, or 28.0 mM glucose for an additional week to compare the effects of prolonged exposure to high glucose on islet recovery and insulin secretory activity. Islets cultured in high glucose (28.0 mM) contained the least amount of recoverable cellular insulin after 1 week of culture ($42.0 \pm 4.7\%$), although a week of culture in 5.6 and 10.0 mM glucose also significantly reduced cellular insulin content (63.2 ± 6.4 and $55.8 \pm 3.8\%$ respectively; Table 1). Similarly, β -cell insulin content decreased dramatically following a week of culture in 28.0 mM glucose compared with 5.6 or 10.0 mM glucose ($P < 0.05$; Table 1). The decrease in insulin content after incubation at 28.0 mM glucose was accompanied by increased cellular DNA recovery compared with islets cultured at 5.6 or 10.0 mM glucose ($P < 0.05$; Table 1). After a week of culture at different glucose concentrations, islets from each group were returned to physiological glucose (5.6 mM) for two additional days of culture. During these 2 days, islet cellular insulin content was partially restored as both the total amount of recoverable islet and individual β -cell insulin contents increased (Table 1).

To evaluate the insulin secretory activity of islets after culture at different glucose concentrations, we compared the

Table 1 Recovery of cellular insulin and DNA content as well as β -cell insulin content after culture at different glucose concentrations

	Medium glucose (mM)	% Recovery		Insulin content (pg per β -cell)
		Insulin	DNA	
Week 1	5.6	100.0 ^a	100.0 ^b	14.3 ± 1.6
Week 2	5.6	63.2 ± 6.4	82.8 ± 4.1	9.9 ± 1.5
	10.0	55.8 ± 3.8	73.0 ± 2.1	9.1 ± 2.1
	28.0	42.0 ± 4.7	104.8 ± 3.2^c	3.7 ± 0.7^d
2 Day	5.6–5.6	59.5 ± 5.4	77.7 ± 1.6	10.2 ± 2.2
Recovery	10.0–5.6	62.2 ± 4.4	78.0 ± 0.7	10.3 ± 1.9
	28.0–5.6	68.4 ± 9.5	80.1 ± 1.9	7.2 ± 2.0

Cellular insulin (5065.9 ± 670.0 ng) and DNA (12.5 ± 0.2 μ g) content values are expressed as recoveries of week 1 values arbitrarily set at 100.0%. Insulin content was calculated by dividing the total insulin content of islets by the number of β -cells ($n = 3$). ^a $P < 0.05$ versus week 2 and 2 days recovery values. ^b $P < 0.05$ versus week 2–5.6 and 10.0 mM and 2 days recovery values. ^c $P < 0.05$ versus week 1, 2 and 2 days recovery values. ^d $P < 0.05$ versus week 1, 2 and 2 days recovery. Data are mean $\pm$ S.E.M.

percentage of insulin released by islets after stimulation with low (2.8 mM) or high (20.0 mM) glucose. Following the first week of culture in 5.6 mM glucose, islets exhibited a stimulation index of 3.9 ± 0.1 in response to a 20.0 mM glucose challenge (Table 2). The insulin released per β -cell in response to a high glucose challenge was $5.5 \pm 0.7 \times 10^{-4}$ ng, or $4.0 \pm 0.2\%$ of the total intracellular insulin stores. After exposing islets to 10.0 mM glucose for 1 week, the insulin secretory response to 20.0 mM glucose ($9.4 \pm 0.2\%$ insulin release) was significantly higher compared with islets cultured at 5.6 or 28.0 mM glucose for 1 week ($6.2 \pm 0.3\%$ vs $7.7 \pm 0.2\%$ respectively; $P < 0.05$; Table 2). However, the stimulation indices of islets were similar after culture at 5.6 and 10.0 mM glucose (3.4 ± 0.4 vs 3.9 ± 0.0 respectively), despite a non-statistically significant higher basal insulin release after culture in 10.0 mM glucose (1.8 ± 0.1 and $2.4 \pm 0.1\%$ respectively; Table 2). Culture in 28.0 mM glucose for 1 week caused significantly increased basal insulin secretion ($3.0 \pm 0.3\%$), while only slightly reducing stimulated insulin release in response to 20.0 mM glucose ($7.7 \pm 0.2\%$, $P < 0.05$, Table 2), resulting in a significantly reduced ($P < 0.05$) stimulation index (2.6 ± 0.2) compared with islets cultured at 5.6 and 10.0 mM glucose.

Despite there being no change in β -cell insulin content after culture in either 5.6 or 10.0 mM glucose (9.9 ± 1.5 and 9.1 ± 2.1 pg respectively), islets cultured in 10.0 mM glucose were more responsive to a glucose challenge and hence released more insulin compared with those cultured at 5.6 mM (9.2 ± 1.7 vs $4.3 \pm 0.2 \times 10^{-4}$ ng respectively; $P < 0.05$; Table 2). Islets, which were cultured in 28.0 mM glucose, contained the least cellular insulin content (3.7 ± 0.7 pg/ β -cell), and thus released even less insulin ($2.5 \pm 0.6 \times 10^{-4}$ ng) upon glucose stimulation.

Stimulation indices of islet from all groups were restored to similar values after the 2-day recovery period (5.6 mM glucose) compared with before, but were also lower due to

Table 2 Glucose-stimulated insulin release after culture at different glucose concentrations

Medium glucose (mM)		Insulin secretory activity (% content)		Insulin release /β-cell (×10 ⁻⁴ ng)		Stimulation index
		2.8 mM	20.0 mM	2.8 mM	20.0 mM	
Week 1	5.6	1.0±0.2 ^a	4.0±0.2	1.7±0.1	5.5±0.7	3.9±0.1 ^e
	5.6	1.8±0.1	6.2±0.3	1.7±0.3	4.3±0.2	3.4±0.4 ^f
Week 2	10.0	2.4±0.1	9.4±0.2 ^b	2.4±0.1	9.2±1.7 ^d	3.9±0.0 ^g
	28.0	3.0±0.3	7.7±0.2 ^c	1.0±0.1	2.5±0.6	2.6±0.2
2 Day	5.6–5.6	2.6±0.2	4.2±0.1	2.9±0.3	4.9±0.8	1.6±0.1
Recovery	10.0–5.6	2.8±0.1	4.5±0.3	2.7±0.4	4.2±0.6	1.6±0.3
	28.0–5.6	3.1±0.8	5.0±1.0	1.8±0.3	3.1±0.7	1.7±0.1

A 120 min *in vitro* static glucose-stimulated insulin release assay was conducted after culture at different glucose concentrations. The stimulation indices were calculated by dividing the amount of insulin released at high glucose (20.0 mM) by that released at low glucose (2.8 mM; *n* = 3). ^a*P* < 0.05 vs 28.0, 28.0–5.6 mM. ^b*P* < 0.05 versus all except 28.0 mM. ^c*P* < 0.05 versus all except 5.6, 10.0 mM. ^d*P* < 0.05 vs 5.6, 28.0, 10.0–5.6, 28.0–5.6 mM. ^{e,f,g}*P* < 0.05 vs 28.0, 5.6–5.6, 10.0–5.6, 28.0–5.6 mM. Data are mean ± S.E.M.

increased basal insulin release as well as reduced stimulated insulin release (Table 2).

Effect of high glucose on neonatal porcine β-cell differentiation, proliferation, and apoptosis

The porcine β-cell mass is dynamic and can increase by both precursor differentiation and β-cell replication (Trivedi *et al.* 2001, Bock *et al.* 2003). To examine the effect of high glucose on β-cell differentiation, we assessed the proportion of CK7-positive ductal epithelial cells in islets exposed to different glucose concentrations *in vitro*. Islets contained 30–35% CK7-positive cells after 7 days of culture in either 5.6 or 10.0 mM glucose. This percentage was reduced to 18.8±1.7% after culture in 28.0 mM glucose, while the percentage of β-cells increased to 29.3±0.6% (Fig. 1).

Neonatal porcine islet cell proliferation has been shown to occur both *in vitro* (Nielsen *et al.* 2003) and *in vivo* (Trivedi *et al.* 2001), and was assessed here after exposure to different glucose concentrations for 7 days. To determine the percentage of proliferating β-cells, dispersed neonatal porcine islet cells were immunostained for both insulin and PCNA. PCNA protein expression is strictly correlated to cell proliferation and the active phases of the cell cycle (Maga & Hubscher 2003). The number of PCNA-positive cells (30%) was similar to the number of Ki-67-positive cells (25%) reported in neonatal porcine islets after 7 days culture in 10 mM glucose (Nielsen *et al.* 2003). By day 14 of culture in 5.6 mM glucose, the percentage of both the total population of PCNA-positive islet cells and PCNA-positive β-cells had dropped significantly by 32.3±3.7 and 53.3±7.4% respectively (*P* < 0.05; Fig. 1b). Culture in 10.0 and 28.0 mM glucose did little to affect the percentage of proliferating islet and β-cells significantly compared with 5.6 mM glucose (Fig. 1b).

Next, neonatal porcine islet cell apoptosis was examined using the TUNEL method to identify DNA fragmentation (Fig. 2). Islet cell apoptosis was assessed in intact islets embedded in agarose using the TUNEL method and co-stained for insulin to identify apoptotic β-cells. Repre-

sentative islets examined after 14 days culture in 5.6 mM glucose showed extensive TUNEL labeling (Fig. 2a). Only few of the islet cells undergoing apoptosis are insulin-positive (Fig. 2b and c). Both whole islet and β-cell apoptosis were not significantly increased after exposure to high glucose concentrations (28.0 mM), 1 week in culture (Fig. 2d).

Discussion

The data obtained in the present study suggest that neonatal porcine islets exposed to high glucose concentrations *in vitro* leads to a proportional increase in β-cells, with only a moderate effect on β-cell function and survival. Exposure to elevated glucose concentrations in culture decreased the insulin content of neonatal porcine islets in a way similar to that described for fetal porcine and adult human islets in tissue culture (Korsgren *et al.* 1989, Marshak *et al.* 1999). In addition to the observed β-cell degranulation, function was also modified by prolonged *in vitro* exposure to different glucose concentrations. Islets cultured in 10.0 mM glucose exhibited higher insulin secretion when compared with those exposed to either 5.6 or 28.0 mM glucose. However, in these islets, the insulin secretion in response to 2.8 mM glucose was also increased resulting in a stimulation index comparable with that for islets cultured in 5.6 mM glucose. Exposure to 28.0 mM glucose also increased basal insulin release and reduced stimulated insulin release at 20.0 mM glucose, thereby resulting in a significantly decreased stimulation index.

Restoration of the glucose concentration to normal (5.6 mM) for 2 days, restored the stimulation index to levels similar to those for islets which had been previously cultured in 5.6 and 10.0 mM glucose, suggesting the detrimental effects of high glucose are reversible. One reason for this may be the increased insulin biosynthesis which occurs in β-cells that are allowed to recover in 5.6 mM glucose following chronic stimulation for 1 week in 28.0 mM glucose. Increased insulin biosynthesis after culture at 5.6 mM glucose could account for the greater insulin content and release per cell seen after the recovery period.

Figure 1 Effect of high glucose culture on neonatal porcine β -cell differentiation and proliferation. (a) Neonatal porcine islets were cultured in 5.6 mM glucose for 7 days and then switched to either 10.0 or 28.0 mM glucose for an additional 7 days. The percentage of β -cells in islets cultured for 7 days in 28.0 mM glucose had significantly increased, while the percentage of CK7-positive cells decreased ($n=3$). * $P<0.05$ vs insulin-positive cells cultured in 5.6-d7-d14, 10-d14, 28-d14. † $P<0.05$ vs CK7-positive cells cultured in 5.6-d1, d7, d14, 10-d14. * $P<0.05$ vs insulin-positive cells cultured in 5.6-d1, d7, d14, 10-d14. (b) Proliferating islet cells and β -cells decreased at the end of 2 weeks in culture in 5.6 mM glucose. High glucose culture had no effect on the percentage of proliferating cells ($n=3$). * $P<0.05$ vs proliferating islet (β and non- β -cells) and β -cells after culture in 5.6 mM glucose for 1 and 7 days. † $P<0.05$ vs proliferating islet cells after culture in 5.6 mM glucose for 1 and 7 days.

Figure 2 Effect of high glucose culture on neonatal porcine β -cell apoptosis. (a–c) Representative islets cultured for 14 days in 5.6 mM glucose were examined for apoptosis using a fluorescent DNA fragmentation assay (TUNEL stain). Microscopic fields of islets were photographed individually to detect apoptotic cells (a) and β -cells (b) and merged to detect apoptotic β -cells (c). Magnification, $\times 200$.

It is also noteworthy that the high glucose exposure in the present study induced more severe impairments in adult human β -cell function (Eizirik *et al.* 1992) than what has been observed for neonatal porcine β -cells in this study. One possible reason to explain this difference may be due to the presence of nicotinamide used in the neonatal islet culture medium. Nicotinamide has been shown to protect against the cytotoxic action of streptozotocin on β -cells as well as significantly increase glucose-stimulated insulin secretion (Sandler *et al.* 1983, Korbutt *et al.* 1996). When cultured with nicotinamide, neonatal porcine islets have been shown to be protected from β -cell desensitization following prolonged exposure to high glucose (Ohgawara *et al.* 1993). The use of nicotinamide in culture media in this study may explain why islets cultured at 28.0 mM glucose still managed to respond with increased insulin release in

response to a 20.0 mM glucose stimulation. The Hams F10 medium used in this study contains niacin, a derivative of nicotinamide, and is also supplemented with additional nicotinamide to a final concentration of 10.0 mM. In many of the studies examining the effects of high glucose concentrations on adult human islets, media was used which either contained no (Eizirik *et al.* 1992, Hostens *et al.* 1999, Marshak *et al.* 1999, Federici *et al.* 2001) or very little (2.0 mM) nicotinamide (Ling & Pipeleers 1996).

High glucose concentrations in culture can influence precursor cell differentiation into β -cells with only a minimal effect on cell replication and apoptosis. *In vivo* evidence in support of the role of hyperglycemia on precursor cell differentiation was reported by Yoon *et al.* (1999). Their group as well as ours (unpublished observation) have noticed a greater tendency for neonatal porcine islet grafts to develop a

more substantial β -cell mass when implanted into diabetic (hyperglycemic) animals compared with non-diabetic (normoglycemic) recipients (Yoon *et al.* 1999). Recent studies have shown that adult stem cells cultured in high glucose medium are able to transdifferentiate into insulin-producing cells (Yang *et al.* 2002, Cao *et al.* 2004, Tang *et al.* 2004). The ability of islets in postnatal life to respond to hyperglycemia by increased precursor cell differentiation has previously been reported to occur *in vivo* (Bonner-Weir *et al.* 1989).

In addition to precursor differentiation, replication of preexisting β -cells has been reported to contribute to the increased β -cell mass seen in transplanted neonatal porcine islet grafts (Trivedi *et al.* 2001). The lack of an effect of high glucose on islet cell replication contradicts the results of King & Chick (1976) who found that elevated glucose concentrations (16.5–27.5 mM) increased the frequency of replicating neonatal rat β -cells in culture. However, one study reported that high glucose (28 mM) was ineffective and may even inhibit β -cell proliferation in islets from 3-day-old neonatal rats (Kaung 1983). Taken together, these results may suggest that the effects of high glucose on β -cell proliferation and differentiation are species-dependent.

Quite possibly, the most significant result of this study is the demonstration that high glucose does not significantly increase the percentage of apoptotic cells in neonatal porcine islets. Unlike adult human islets which contain 48% apoptotic β -cells after exposure to 16.7 mM glucose for 5 days (Federici *et al.* 2001), neonatal porcine islets contained <5% apoptotic β -cells even after exposure to 28.0 mM glucose for 7 days. Despite the low number of apoptotic β -cells, the percentage of total islet cells undergoing apoptosis was approximately 30%, which has also been reported by others (Yoon *et al.* 1999). This high percentage of cell death, which is present even at the beginning of the culture period (day 1), has been attributed to the isolation procedure since 30% of islet cells have been reported to be undergoing apoptosis immediately following the isolation and culture of both human and porcine pancreases (Paraskevas *et al.* 2000, Stadlbauer *et al.* 2003). The ability of the neonatal porcine islet cells to resist the toxic effects of high glucose is of paramount importance for their ability to survive posttransplantation. Sustained exposure to hyperglycemia in the posttransplant environment can contribute to increased β -cell apoptosis and reduced β -cell mass (Biarnes *et al.* 2002). While many of these studies were performed using adult islets, donor age can significantly affect cell survival. Neonatal islets are known to be more robust and to survive better in tissue culture compared with fragile adult islets. This robustness may be due to the metabolic immaturity of neonatal islets which generate lower ATP and thus a lower secretory response to glucose compared with adult islets (Boschero *et al.* 1990). Neonatal islets are also known to be better protected than adult islets against oxidative stress due to their more active peroxidase system (Stoppiglia *et al.* 2002). Indeed, we have found significantly higher expression levels of X-linked inhibitor of apoptosis, which is known to protect against apoptosis, in neonatal compared with adult porcine islets (unpublished

observation, Plesner *et al.* 2005). High glucose has also been shown to have a beneficial effect on neonatal islets by enhancing the H_2O_2 scavenger system, a phenomenon not observed in adult islets (Stoppiglia *et al.* 2002).

Based on *in vivo* data, which found that exposure to hyperglycemia can increase the β -cell mass of neonatal porcine islet grafts by β -cell differentiation from CK7-positive cells, we have now confirmed this observation *in vitro* (Korbitt *et al.* 1996, Trivedi *et al.* 2001, Rayat *et al.* 2003). Of major significance is the fact that β -cell apoptosis does not appear to increase in frequency following exposure to high glucose, which is in contrast to the situation with adult human islets. Given the ease by which neonatal porcine islets can be obtained and their enormous growth potential, these insulin-producing cells are a potentially useful source of insulin-producing tissue for clinical transplantation.

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