

High glucose-induced impairment in insulin secretion is associated with reduction in islet glucokinase in a mouse model of susceptibility to islet dysfunction

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Abstract

Type 2 diabetes is characterized by islet dysfunction resulting in hyperglycemia, which can then lead to further deterioration in islet function. A possible mechanism for hyperglycemia-induced islet dysfunction is the accumulation of advanced glycation end products (AGE). The DBA/2 mouse develops pancreatic islet dysfunction when exposed to a high glucose environment and/or obesity-induced insulin resistance. To determine the biochemical cause of dysfunction, DBA/2 and C57BL/6 control islets were incubated in 11.1 mM or 40 mM glucose in the absence or presence of the AGE inhibitor aminoguanidine (AG) for 10 days. Basal (2.8 mM glucose) insulin release was increased in both DBA/2 and C57BL/6 islets incubated with 40 mM vs 11.1 mM glucose for 10 days. Chronic exposure to hyperglycemia decreased glucose (20 mM)-stimulated insulin secretion in DBA/2 but not in C57BL/6 islets. AG significantly increased fold-induced insulin release in high glucose cultured DBA/2 mouse islets, but did not affect C57BL/6 islet function. DBA/2 islet glucokinase was significantly reduced following 40 mM glucose culture, compared with 11.1 mM glucose cultured DBA/2 islets and 40 mM glucose cultured C57BL/6 islets. Incubation of islets with AG resulted in a normalization of DBA/2 islet glucokinase levels. In conclusion, chronic high glucose-induced increases in AGE can result in islet dysfunction and this is associated with reduced glucokinase levels in a mouse model with susceptibility to islet failure.

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Introduction

Reduced glucose-mediated insulin release is a characteristic feature of type 2 diabetes resulting in hyperglycemia (Porte 1991). Furthermore, both *in vivo* (Leahy *et al.* 1987, Bedoya & Jeanrenaud 1991) and *in vitro* (Davalli *et al.* 1991, Robertson *et al.* 1992) studies have shown that hyperglycemia itself can activate pathways which diminish glucose-mediated insulin release. Thus, this has the possibility of creating a feedforward cycle of increasing plasma glucose levels leading to impaired insulin secretory function, further worsening hyperglycemia.

Although high glucose-induced impairment in insulin secretion is well recognized, the mechanisms causing this phenomenon are not well understood. For example, it has been proposed that chronic high-glucose-induced impairment in β -cell function can result from 'β-cell exhaustion' (Sako & Grill 1990, Kaiser *et al.* 1991, Eizirik *et al.* 1992, Ling *et al.* 1996), although this has not been a consistent finding (Davalli *et al.* 1991). Another

possible mechanism is a reduction in insulin gene transcription factors (e.g. PDX-1 and RIPE3b1) leading to decreased β -cell insulin content (Robertson *et al.* 1992, Poitout *et al.* 1996, Moran *et al.* 1997, Marshak *et al.* 1999). However in at least one study, reduction of the transcription factor PDX-1 using a specific antisense oligodeoxynucleotide did not lead to decreased insulin levels (Kajimoto *et al.* 1997).

Advanced glycation end products (AGEs) accumulate in cells and tissues as a result of chronic high glucose exposure and are thought to be an important mechanism in the pathogenesis of diabetic complications (Forbes *et al.* 2003). It has been proposed that the *in vitro* accumulation of AGEs may lead to impaired β -cell function (Matsuoka *et al.* 1997, Tajiri *et al.* 1997, Kajimoto *et al.* 1999, Kaneto *et al.* 1999). Proposed AGE-related defects in insulin secretion include reduced insulin biosynthesis (Tajiri *et al.* 1997) as a result of diminished insulin gene promoter activity (Matsuoka *et al.* 1997) and reduced β -cell mass (Piercy *et al.* 1998, Kaneto *et al.* 1999).

The DBA/2 and the C57BL/K_sJ mouse strain, which carries a small portion of the DBA/2 genome (Naggert *et al.* 1995), have a genetic predisposition to pancreatic islet failure. Islet function is grossly impaired in DBA/2 and C57BL/K_sJ mice expressing the *db/db* gene (which results in obesity and insulin resistance as a result of a defective leptin receptor molecule) with an absence of both first and second phase insulin secretion. The hyperglycemia which develops is a consequence of decreased insulin production from a reduction in the islet β -cell population (Baetens *et al.* 1978, Leiter *et al.* 1981, Molina *et al.* 1984). By contrast, expression of the *db/db* gene on a C57BL/6 genetic background results in marked obesity, insulin resistance, with only mild hyperglycemia and marked hyperinsulinemia (Leiter *et al.* 1981). Furthermore, when islets from C57BL/K_sJ mice are exposed to a high glucose environment by intrasplenic transplantation in syngeneic donors made hyperglycemic by treatment with streptozotocin, they show a depressed rate of cell proliferation and are eliminated within 12 days (Leiter 1987). On the other hand, C57BL/K_sJ islets transplanted into normoglycemic donors are retained. Moreover, chronic incubation of DBA/2 mouse islets with glucose concentrations greater than 11.1 mM caused diminished glucose-induced insulin secretion (Pehuet-Figoni *et al.* 1994). These studies highlight the increased susceptibility of islets from the DBA/2 and C57BL/K_sJ strains to a chronic high glucose milieu. However, a mechanism for this pancreatic islet failure has not been explored.

The aim of the present study was to investigate differences in early biochemical defects in the failure-prone DBA/2 mouse compared with C57BL/6 mice using isolated islets cultured in a high glucose environment.

Materials and methods

Materials

Collagenase P was obtained from Boehringer Mannheim (Mannheim, Germany). Culture medium RPMI 1640 with L-glutamine without sodium bicarbonate was obtained from Trace Scientific Ltd (Victoria, Australia). Aminoguanidine was obtained from Sigma-Aldrich (Australia).

Animals

Male C57BL/6 and DBA/2 mice were purchased from the Walter and Eliza Hall Institute Animal Research Facility (Kew, Victoria, Australia) and housed in the Department of Medicine Animal Research Facility under a 12 hour light/dark cycle with a standard laboratory non-purified diet containing 77% of energy as carbohydrate, 20% protein and 3% of calories as fat

(Barastock Products, Pakenham, Australia) provided *ad libitum* unless otherwise stated. All animals were 8–10 weeks of age at the time of study and all procedures described below were approved by the Royal Melbourne Hospital Animal Research Ethics Committee.

Islet isolation and culture

Pancreatic islets were isolated by collagenase digestion as previously described (Kooptiwut *et al.* 2002, Zraika *et al.* 2002). The isolation of the islets was accomplished using a Ficoll gradient and hand picking under a stereomicroscope. Islets were cultured in RPMI 1640 containing either 11.1 mM or 40 mM glucose with 10% heat inactivated fetal calf serum, 100 U/ml penicillin, and 100 μ g/ml streptomycin at 37 °C in humidified air 5% CO₂ for 10 days, with the media being changed every second day. Aminoguanidine at a final concentration of 1 mM was added to the media of half of the islets incubated with 40 mM glucose for 10 days.

Insulin secretion assay

Islets were washed twice in Krebs Ringer Bicarbonate Buffer (KRB) (111 mM NaCl, 4.8 mM KCl, 2.3 CaCl₂, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 25 mM NaHCO₃, pH 7.4), 10 mM Hepes, 2.8 mM glucose, 0.2% BSA fraction V (Sigma) and preincubated in the same buffer for 90 min at 37 °C in humidified air 5% CO₂. Triplicate batches of five islets were transferred to borosilicate tubes containing 1 ml of KRB supplemented with either 2.8 mM glucose, 20 mM glucose or 20 mM glucose plus 275 μ M tolbutamide and incubated for 60 min at 37 °C. The medium was collected after gentle centrifugation and stored at –20 °C for measurement of insulin.

Measurement of advanced glycation end products

The AGE-associated fluorescence (Ex 370 nm, Em 440 nm) was estimated in media samples following enzymatic hydrolysis with proteinase K (10 μ g/ml). Samples were then analyzed using an on-line spectrofluorometric detection flow system as previously described by our group (Forbes *et al.* 2002). Results were expressed in arbitrary units (AU) corrected for the protein/peptide content of each sample estimated by an on-line absorbance detector (A 280).

Islet insulin content

Islets were lysed in ice cold acid-ethanol (2% HCl in 95% ethanol) and placed at –20 °C for at least 1 h. The samples were thawed and sonicated on ice for about

20 min to complete the lysis. The supernatants were collected after gentle centrifugation (900 *g* for 5 min at 4 °C) and kept at -70 °C until measurement of insulin content by radioimmunoassay.

Real-time quantitative RT-PCR

Gene expression of mouse pro-insulin II in isolated pancreatic islets was determined using real-time quantitative RT-PCR performed using the TaqMan system (ABI Prism 7700, Perkin-Elmer Inc, PE Biosystems, Foster City, CA, USA), as previously described (Tikellis *et al.* 2003, 2004). Total RNA was extracted from freshly isolated pancreatic islets following homogenisation using the Ultra-Turrax (Janke & Kunkel IKA, Labortechnik, Germany) in TRIZOL (Life Technologies Inc, Gaithersburg, MD, USA). cDNA was then synthesized with a reverse transcriptase reaction carried out using standard techniques (Superscript First Strand Synthesis System for RT-PCR, Life Technologies Inc) with random hexamers, dNTPs and total RNA. To assess genomic DNA contamination, controls without reverse transcriptase were included. The oligonucleotides and probe for mouse pro-insulin II were designed using the software program, 'Primer Express' (PE Applied Biosystems, Foster City, CA, USA) and are 3' oligonucleotide (5'-TCAAGCAGCACCTTTGTG GTT), 5' oligonucleotide (5'-GGGACATGGGTGTGT AGAAGAAG) and probe (FAM5'-CCCACACACCAG GTAG-MGB).

The RT-PCR multiplex reaction took place with 500 nmol/l of forward and reverse primer and 50 nmol/l of FAM/MGB probe and VIC/TAMRA 18S ribosomal probe, in Taqman universal PCR master mix (PE Biosystems, Foster City, CA, USA). Amplification of 18S was used to correct for loading. Each sample was run and analyzed in triplicate.

Measurement of glucose phosphorylating activity

Approximately 300 islets were washed with 2.8 mM KRB twice and incubated for another 90 min. Islets were homogenized in ice-cold buffer containing 20 mM K₂HPO₄, 1 mM EDTA, 5 mM dithiothreitol and 110 mM KCl. The islet homogenate was then centrifuged at 12 000 *g* for 10 min. The supernatant was used for glucokinase and hexokinase determination by a spectrophotometric assay (Andrikopoulos *et al.* 1993, Kooptiwut *et al.* 2002). The reaction volume contained 100 µl of islet supernatant in 500 µl of 50 mM Hepes/HCl, pH 7.6, 100 mM KCl, 7.4 mM MgCl₂, 15 mM β-mercaptoethanol, 0.50 mM NAD⁺, 0.05% bovine serum albumin, 0.70 unit/ml glucose-6-phosphate dehydrogenase from *Leuconostoc mesenterodes* and 5 mM ATP. The reaction was performed for 90 min

and absorbance measured at 340 nm. The activity of glucokinase was calculated by subtracting the activity of hexokinase, which was determined at a glucose concentration of 0.5 mM from the total phosphorylating activity, measured at 100 mM glucose.

Glucokinase, hexokinase and GLUT2 Western blot analysis

Western blot analyses were performed as previously described (Kooptiwut *et al.* 2002) to quantify glucokinase, hexokinase and GLUT2 protein levels. Following incubation, approximately 400–500 islets were sonicated for 20 min at 4 °C in a lysis buffer containing 5% SDS, 80 mM Tris /HCl, pH 6.8, 5 mM EDTA, 1 mM phenylmethylsulfonylfluoride, 10 µg DNase I, and 0.2 mM N-ethylmaleimide. The lysate was retrieved after centrifugation at 12 000 *g* for 10 min at 4 °C. Protein content was measured by the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA). Samples containing 20 µg of protein were resolved by electrophoresis through a 10% (for glucokinase and GLUT2) or 7.5% (for hexokinase) polyacrylamide gel. Following transfer to PVDF membrane glucokinase was immunodetected using a specific anti-glucokinase antibody (cat # sc-7908, Santa Cruz Biotech. Inc, Santa Cruz, CA, USA) at a 1:500 dilution followed by incubation with swine anti-rabbit IgG at 1:2500 dilution. Hexokinase was immunodetected using a specific anti-hexokinase antibody (cat # sc-6517, Santa Cruz Biotech. Inc) at a 1:500 dilution followed by incubation with swine anti-goat IgG at 1:2500 dilution. GLUT2 was immunodetected using a specific anti-GLUT2 antibody (cat # sc-9117, Santa Cruz Biotech. Inc) at a 1:2000 dilution followed by incubation with swine anti-rabbit IgG at 1:2500 dilution. Protein bands were visualized by enhanced chemiluminescence (Renaissance, NEN Life Science Products Inc, Boston MA, USA) and quantified using scanning densitometry.

Cell viability assay

Viable cell ratios were determined at each time-point using the dye exclusion assay (Altman *et al.* 1993). This assay is based on the exclusion of trypan blue dye from viable cells. Ten islets were picked and treated with trypsin/EDTA for 15–20 min at 37 °C. The cell suspension was then centrifuged at 900 *g* for 10 min at 4 °C and the pellet resuspended in Hanks' Balanced Salt Solution (HBSS). Cells were then incubated with trypan blue (final concentration, 0.2 mg/ml) for 3–5 min and observed under a microscope. Viable (unstained) and non-viable (stained) cells were counted with a hemocytometer and the viable cell ratio calculated.

Statistical Analysis

Insulin secretion results were corrected for and expressed as the percent of total islet insulin content. Data is presented as mean \pm S.E.M. for the number indicated. Statistical analysis was performed using the Mann–Whitney *U* test and the Wilcoxon paired test. A $P < 0.05$ was considered significant.

Results

Islet culture in 11.1 mM or 40 mM glucose for 10 days

Isolated DBA/2 and C57BL/6 mouse islets were cultured in 11.1 mM glucose for 10 days, insulin release was assessed at basal (2.8 mM) and stimulating (20 mM) glucose concentrations. Fig. 1A shows that insulin release was stimulated with 20 mM glucose from both DBA/2 and C57BL/6 mouse islets. As we have previously shown (Kooptiwut *et al.* 2002) insulin release was increased from DBA/2 compared with C57BL/6 islets at both 2.8 mM and 20 mM glucose.

Incubation of the islets with 40 mM glucose for 10 days increased basal insulin release in both DBA/2 (35.2 ± 5.8 vs $1.2 \pm 0.2\%$ content, $P < 0.05$) and C57BL/6 (17.9 ± 6.0 vs $0.3 \pm 0.1\%$ content, $P < 0.05$) mice but the increase was more marked in DBA/2 compared with C57BL/6 islets ($P < 0.05$). When stimulated with 20 mM glucose insulin secretion was significantly increased from C57BL/6 islets while there was no change from DBA/2 islets when compared with 2.8 mM glucose condition (Fig. 1A). However, when challenged with 20 mM glucose + 275 μ M tolbutamide, there was a significant increase in insulin release from DBA/2 islets ($P < 0.05$). This suggests a functional defect in DBA/2 islets when chronically incubated with 40 mM glucose.

To determine the effects of advanced glycation end products on secretory function, aminoguanidine was added to DBA/2 and C57BL/6 islets cultured for 10 days in 40 mM glucose. Incubation of islets in the presence of 1 mM aminoguanidine significantly reduced basal insulin secretion from DBA/2 (35.2 ± 5.8 vs $5.9 \pm 1.0\%$ content, $P < 0.05$) and C57BL/6 (17.9 ± 6.0 vs $2.5 \pm 0.5\%$ content, $P < 0.05$) mouse islets (Fig. 1A). Furthermore, insulin release in response to 20 mM glucose was significantly higher from both DBA/2 and C57BL/6 islets compared with the respective 2.8 mM glucose condition. This suggests that aminoguanidine improves insulin secretory function in DBA/2 mouse islets.

When the insulin secretion was expressed as a fold above basal (2.8 mM glucose), the stimulation in response to glucose was similar between the two strains of mice when cultured at 11.1 mM glucose for 10 days

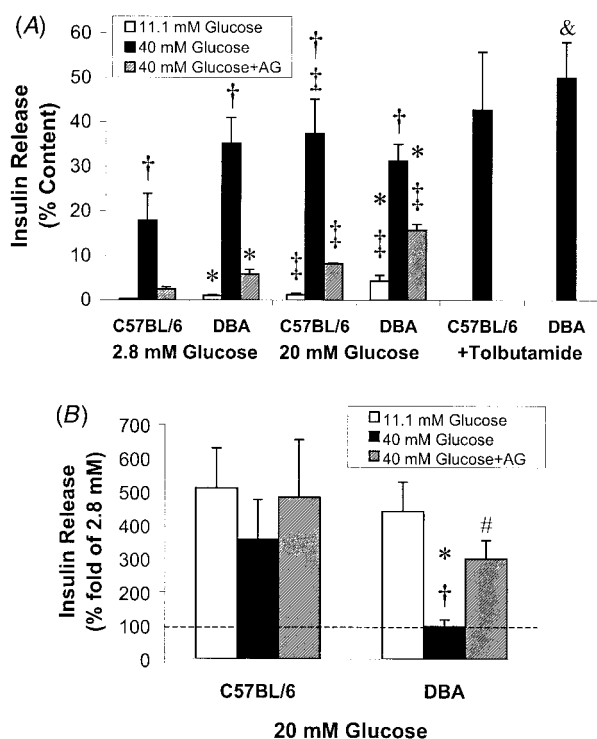


Figure 1 (A) Glucose-mediated insulin secretion in islets incubated with 11.1 mM glucose or 40 mM glucose in the absence or presence of 1 mM aminoguanidine (AG) for 10 days. Islets from 8–10 week-old DBA/2 and C57BL/6 mice were isolated as described in the Materials and Methods section and batches of five islets preincubated in KREBS buffer containing 2.8 mM glucose for 90 minutes. Insulin secretion was assessed at 2.8 mM glucose, 20 mM glucose or 20 mM glucose + 275 μ M tolbutamide over a 60 min period. (B) Insulin secretion expressed as fold increase of the result obtained with 2.8 mM glucose. Results are presented as means \pm S.E.M. ($n = 6$). * $P < 0.05$ compared with C57BL/6 mouse islets, # $P < 0.05$ compared with islets incubated with 1 mM aminoguanidine, † $P < 0.05$ compared with islets incubated in 11.1 mM glucose for 10 days, ‡ $P < 0.05$ compared with islets stimulated with 2.8 mM glucose for 60 minutes, & $P < 0.05$ compared with islets stimulated with 20 mM glucose for 60 minutes.

(Fig. 1B) indicative of comparable islet function. In contrast, following culture at 40 mM glucose for 10 days, despite an increase in basal insulin secretion in both strains, fold stimulation of insulin secretion was completely blocked in DBA/2 mouse islets compared with 11.1 mM glucose for 10 days but not in C57BL/6 islets, illustrating the susceptibility of DBA/2 mouse islets to a high glucose milieu. Aminoguanidine significantly increased fold-induced insulin release in high glucose cultured DBA/2 mouse islets, but had no effect in fold stimulation of insulin release from C57BL/6 mouse islets cultured in 40 mM glucose.

Culture in 40 mM glucose for 10 days caused a comparable increase in AGE in both DBA/2 and

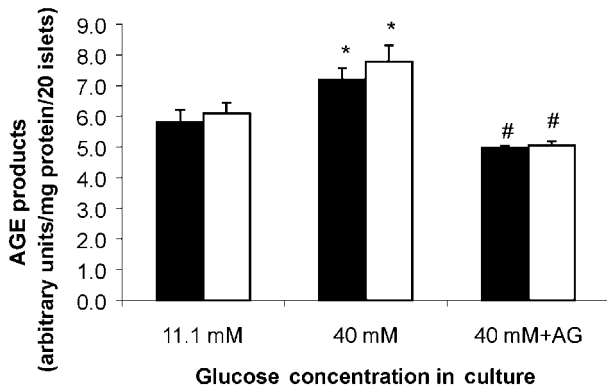


Figure 2 Advanced glycation end products (AGE) (Ex 370 nm, Em 440 nm, normalized to protein content at 280 nm) in DBA/2 (open bars) and C57BL/6 (solid bars) islets incubated in 11.1 mM glucose or 40 mM glucose in the absence or presence of 1 mM aminoguanidine (AG) for 10 days. Results are presented as means \pm S.E.M. ($n=3$). * $P<0.05$ compared with islets incubated in 11.1 mM glucose, # $P<0.05$ compared with islets incubated in 40 mM glucose.

C57BL/6 islets (Fig. 2). Aminoguanidine resulted in a reduction in AGE in both strains of mice.

Islet insulin content and mRNA

Insulin content and mRNA levels in islets cultured with 11.1 mM or 40 mM glucose for 10 days is shown in Fig. 3. There was a marked decrease in islet insulin content following 40 mM glucose compared with 11.1 mM glucose culture in both strains of mice, with no difference in insulin content between DBA/2 and C57BL/6 islets (Fig. 3A). Treatment with aminoguanidine resulted in increased insulin content in islets from both strains of mice incubated with 40 mM glucose for 10 days (Fig. 3A). However, DBA/2 mouse islets contained significantly less insulin following 40 mM glucose plus aminoguanidine treatment compared with similarly treated C57BL/6 mouse islets.

In contrast insulin mRNA was significantly increased in both DBA/2 and C57BL/6 islets following incubation in 40 mM glucose for 10 days (Fig. 3B). Aminoguanidine had no effect on insulin mRNA in either strain compared with 40 mM glucose incubation for 10 days. There was a trend ($P=0.1$) for insulin mRNA to be higher in DBA/2 compared with C57BL/6 islets incubated with 11.1 mM glucose for 10 days (Fig. 3B).

Cell viability

Cell viability was determined under the culture conditions described above and the data shown in Fig. 4. Chronic high glucose culture resulted in decreased cell viability in both strains, being statistically significant at 6,

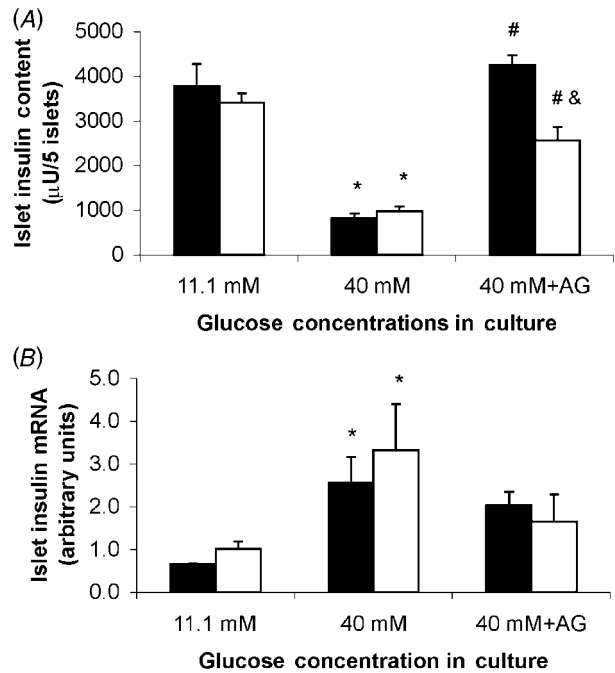


Figure 3 (A) Insulin content in DBA/2 (solid bars) and C57BL/6 (open bars) islets incubated in 11.1 mM glucose or 40 mM glucose in the absence or presence of 1 mM aminoguanidine (AG) for 10 days. Results are presented as means \pm S.E.M. ($n=6$). (B) Insulin mRNA in DBA/2 (solid bars) and C57BL/6 (open bars) islets incubated in 11.1 mM glucose or 40 mM glucose in the absence or presence of 1 mM AG for 10 days. Results are presented as means \pm S.E.M. ($n=3$). * $P<0.05$ compared with islets incubated in 11.1 mM glucose, # $P<0.05$ compared with islets incubated in 40 mM glucose, & $P<0.05$ compared with similarly treated C57BL/6 islets.

8 and 10 days for C57BL/6 (Fig. 4A) and at 8 and 10 days for DBA/2 (Fig. 4B) mouse islets. At 10 days, cell viability was the same between C57BL/6 and DBA/2 mice ($72.6 \pm 1.0\%$ vs $74.0 \pm 1.8\%$, $P=0.52$). Aminoguanidine treatment completely reversed the decrease in cell viability caused by high glucose in both strains of mice (Figs 4A and B).

Islet glucokinase, hexokinase and GLUT2 Levels

A possible mechanism was investigated for the changes in insulin secretion in DBA/2 mouse islets incubated with 40 mM glucose in the presence of comparable insulin content and cell viability between the two strains of mice. To determine whether the altered insulin secretion in DBA/2 islets following chronic high glucose was the result of impaired glucose sensing, the protein levels of glucokinase, hexokinase and GLUT2 were measured and the results shown in Fig. 5. Western blotting on freshly isolated islets showed that glucokinase

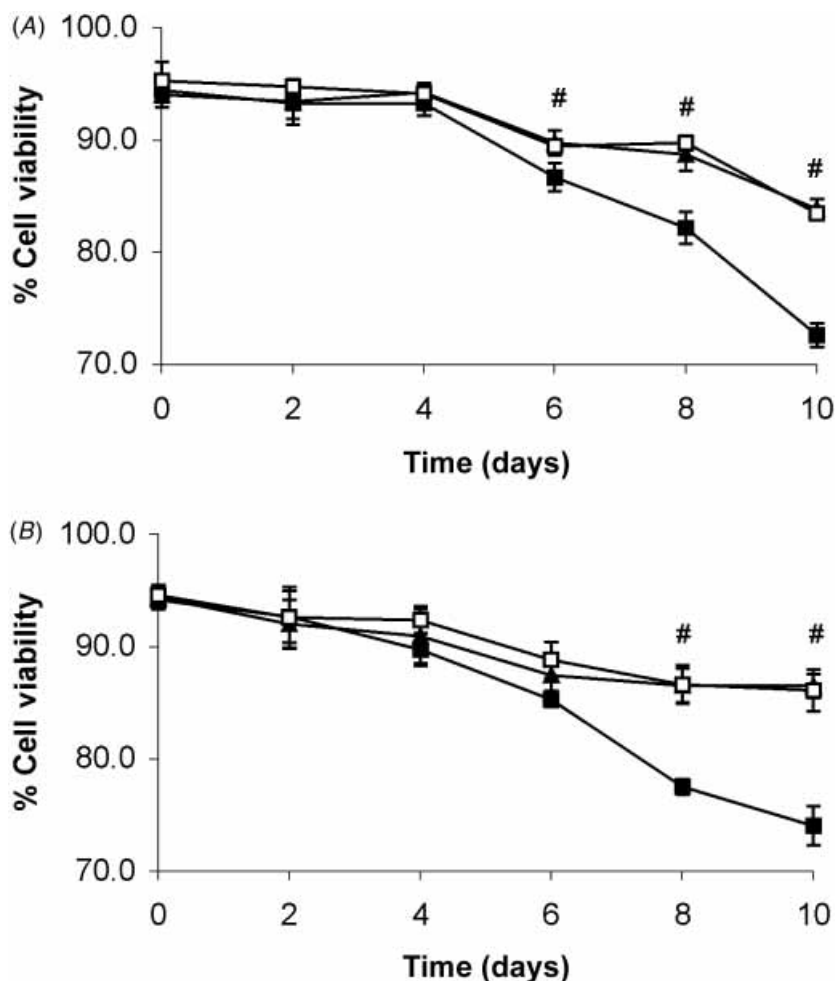


Figure 4 Cell viability determinations in C57BL/6 (A) and DBA/2 (B) islets incubated in 11.1 mM glucose (▲) or 40 mM glucose in the absence (■) or presence (□) of 1 mM aminoguanidine for 10 days. Results are presented as means \pm S.E.M. ($n=3$). # $P < 0.05$ compared with islets incubated in 40 mM glucose.

levels were comparable between the two strains of mice (18.9 ± 0.6 vs 20.0 ± 1.3 arbitrary units C57BL/6 vs DBA/2, $n=3$, $P=0.5$). Glucokinase protein levels were also similar between DBA/2 and C57BL/6 islets following 11.1 mM glucose culture for 10 days. Culture with 40 mM glucose for 10 days resulted in reduced glucokinase in DBA/2 but not C57BL/6 islets. Furthermore, glucokinase protein levels were also 50% lower in DBA/2 mouse islets than that detected in C57BL/6 mouse islets (Fig. 5). In addition glucokinase activity was also decreased in DBA/2 compared with C57BL/6 islets following high glucose culture (2.6 ± 0.5 vs 3.8 ± 0.5 nmol/min/mg protein, $n=6$, $P=0.05$). Aminoguanidine treatment resulted in significantly increased glucokinase protein levels in DBA/2 mouse islets incubated with 40 mM glucose for 10 days (Fig. 5). In contrast, the presence of this drug did not affect

glucokinase protein levels in C57BL/6 mouse islets incubated with 40 mM glucose for 10 days (Fig. 5).

To determine the effect of chronic high glucose on the protein levels of other components of the glucose sensing system, GLUT2 and hexokinase levels were measured and the results shown in Fig. 6. There was no difference in GLUT2 protein levels between the two strains of mice (Fig. 6A). The lack of change in hexokinase activity (3.1 ± 0.3 vs 3.2 ± 0.9 nmol/min/mg protein, $n=6$, $P=0.4$) was also reflected in no change in hexokinase protein levels (Fig. 6B).

Discussion

Glucokinase is the β -cell glucose-sensor for insulin secretion (Steiner & James 1992, Matschinsky 1996,

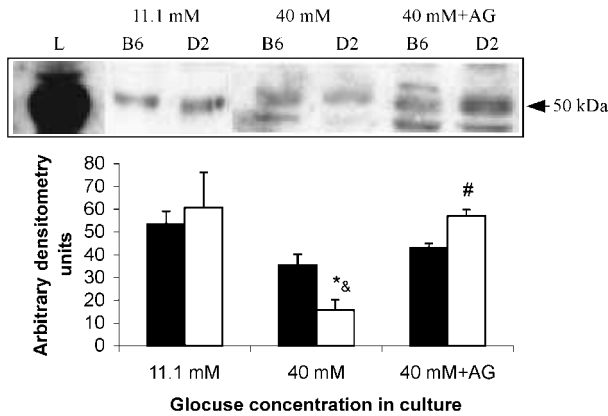


Figure 5 Glucokinase protein levels in DBA/2 (D2, open bars) and C57BL/6 (B6, solid bars) mouse islets incubated with 11.1 mM glucose or 40 mM glucose for 10 days in the absence or presence of 1 mM aminoguanidine (AG). A liver lysate (L) was used as an internal control. The graph is the mean \pm S.E.M. of at least three independent experiments. * $P < 0.05$ compared with islets incubated in 11.1 mM glucose, # $P < 0.05$ compared with islets incubated in 40 mM glucose, & $P < 0.05$ compared with similarly treated C57BL/6 islets.

Schuit *et al.* 2001). Deletion of one allele of β -cell glucokinase by homologous recombination in mice resulted in reduced glucose-mediated insulin release (Terauchi *et al.* 1995, Andrikopoulos *et al.* 2000). Interestingly while stimulated insulin levels were lower in the heterozygous knockout mice, basal insulin levels were not different to wildtype and were trending towards being higher (Terauchi *et al.* 1995, Andrikopoulos *et al.* 2000). In the present study we showed that chronic high glucose incubation caused a 50% decrease in glucokinase protein levels and this was associated with a reduction in glucose-stimulated insulin release from DBA/2 islets. This is in contrast to a previous study which showed an increase in glucokinase activity levels following high glucose culture (Liang *et al.* 1994). The reason for the discrepancy could be due to a difference in strain backgrounds (a hybrid background vs DBA/2 in our study) and the length of time and concentration of glucose (30 mM for 4 days vs 40 mM glucose for 10 days in our study). While chronic 40 mM glucose incubation resulted in increased basal insulin secretion, this occurred in both strains of mice. Furthermore tolbutamide was able to elicit a significant increase in insulin release from DBA/2 islets incubated chronically with 40 mM glucose. Thus while the increase in basal insulin secretion may have been a contributory factor, we believe that the reduction in islet glucokinase was the main cause of the reduction in function of DBA/2 islets. Increases in basal insulin secretion following high glucose culture have previously been shown in rat islets and purified β -cells (Purrello *et al.* 1989, Ling *et al.* 1996, Tajiri *et al.* 1997). Furthermore, we showed that

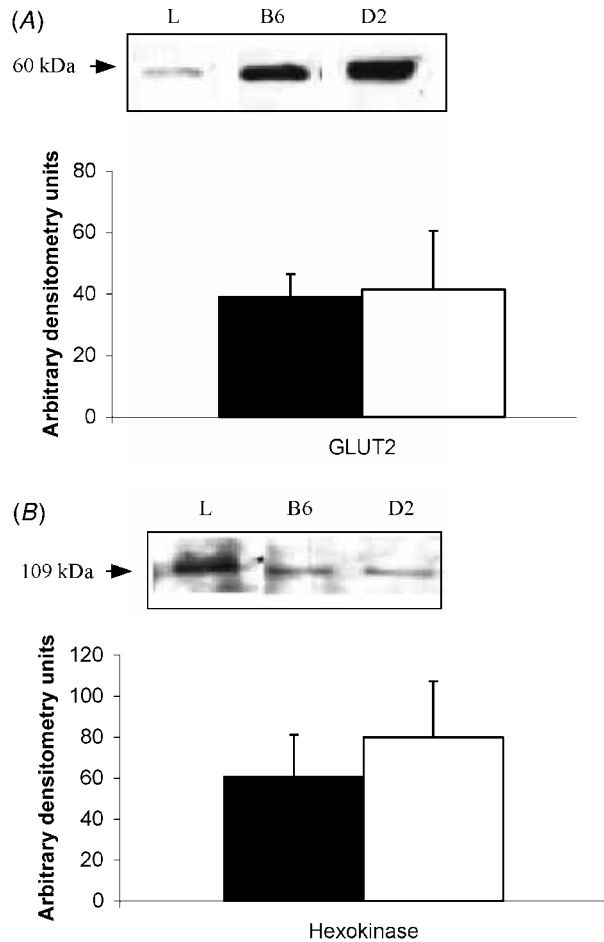


Figure 6 (A) Hexokinase and (B) GLUT2 protein levels in DBA/2 (D2, open bars) and C57BL/6 (B6, solid bars) mouse islets incubated with 40 mM glucose for 10 days in the absence or presence of 1 mM aminoguanidine. A liver lysate (L) was used as an internal control. The graph is the mean \pm S.E.M. of six independent experiments.

aminoguanidine, an inhibitor of the glycation process reduced basal insulin secretion in both DBA/2 and C57BL/6 islets following high glucose culture, but was associated with significant increases in glucokinase levels only in the DBA/2 mouse. This further supports the notion that the effect of high glucose to reduce glucose-mediated insulin secretion from the DBA/2 islet is associated with reduced glucokinase levels.

Formation of advanced glycation end products has been shown to be detrimental to islet function, an effect improved by aminoguanidine (Tajiri *et al.* 1997). This is corroborated by a study in the immortal β -cell line HIT-T15 in which induction of glycation suppressed the activity of the glucokinase promoter resulting in reduced mRNA and protein levels (Kajimoto *et al.* 1999). Furthermore it was shown that the inhibition of glucokinase gene expression was prevented by treatment

with N-acetyl-cysteine or aminoguanidine providing further evidence that increased glycation was involved (Kajimoto *et al.* 1999). While it has been suggested that aminoguanidine may act by inhibiting nitric oxide synthase, evidence suggests that in pancreatic islets the main mechanism of action is via reduction of AGE (Tajiri *et al.* 1997). This study demonstrated that N^G-Methyl-L-arginine, a nitric oxide synthase inhibitor, failed to reproduce the beneficial effects of aminoguanidine in rat islets. It has also been shown that the effects of aminoguanidine on insulin secretion are not via inhibition of glucose oxidation (Eizirik *et al.* 1994). One may also argue that aminoguanidine itself is directly increasing glucokinase levels and thus its effect is independent of glycation inhibition. However, we did not see a similar increase in glucokinase protein levels in control C57BL/6 islets treated with aminoguanidine. It is of interest that the increase in AGE with high glucose culture was similar in DBA/2 and C57BL/6 islets, yet only the former displayed a defect in insulin secretion, associated with reduced glucokinase levels. Furthermore the fact that these phenotypic differences between the two strains were present *in vitro* 10 days after removal from the *in vivo* situation indicates that they are due to genetic variations. This suggests that DBA/2 mice are not able to cope with oxidative stress as well as C57BL/6 mice and this may be due to the inherent susceptibility of DBA/2 islets to develop diabetes when exposed to a permissive environment (Leiter *et al.* 1981, Leiter 1987, Pehuet-Figoni *et al.* 1994).

Other explanations have been proposed for high glucose induced impairments in insulin release. Chronic high glucose incubation has been shown to cause excessive insulin secretion depleting intracellular stores (Sako & Grill 1990, Kaiser *et al.* 1991, Eizirik *et al.* 1992, Ling *et al.* 1996, Bjorklund *et al.* 2000), this effect being prevented by agents, such as diazoxide (Sako & Grill 1990, Bjorklund *et al.* 2000), that inhibit insulin release. It has also been suggested that chronic high glucose resulted in reduced insulin biosynthesis as a result of decreased expression of the insulin gene transcription factors PDX-1 and RIPE-3b1 (Robertson *et al.* 1992, Poutout *et al.* 1996, Moran *et al.* 1997, Marshak *et al.* 1999). Consequently increased D-ribose induced glycation has been shown to cause a decrease in insulin gene promoter activity, which was prevented by aminoguanidine (Matsuoka *et al.* 1997). Moreover, aminoguanidine has been shown to cause an increase in proinsulin biosynthesis while decreasing AGE-related fluorescence in rat islets chronically incubated with 38 mM glucose (Tajiri *et al.* 1997). Lastly, aminoguanidine and other antioxidants have been shown to increase β -cell mass in diabetic C57BL/KsJ-*db/db* mice (Kaneto *et al.* 1999, Piercy *et al.* 1998).

In our study we found that incubation with 40 mM glucose caused reduced insulin content and cell viability

in both DBA/2 and C57BL/6 islets. Aminoguanidine treatment caused a significant increase in islet insulin content and cell viability in cells incubated with 40 mM glucose in both DBA/2 and C57BL/6 islets. The failure to find a difference in cell survival between the DBA/2 and C57BL/6 islets was surprising given the existing data showing a marked difference in survival of these islets when exposed *in vivo* to hyperglycemia (Baetens *et al.* 1978, Leiter *et al.* 1981, Molina *et al.* 1984). The difference could be due to the duration of the exposure to hyperglycemia or to the presence of other factors contributing to cell death in the DBA/2 mice that are present *in vivo* but not *in vitro*. It is likely that a separate mechanism operates in DBA/2 mice exposed to hyperglycemia for longer. However it appears that aminoguanidine can also block this mechanism since it has been shown that this agent prevents β -cell death when administered *in vivo* to C57BL/KsJ-*db/db* mice (Kaneto *et al.* 1999, Piercy *et al.* 1998).

Interestingly we found that islet insulin mRNA was in fact increased following 40 mM glucose culture for 10 days and that aminoguanidine had no further effect in both strains of mice. Thus, the increase in insulin content that we observe in both the DBA/2 and C57BL/6 islets following aminoguanidine treatment is most probably due to a decrease in chronic secretion or an increase in cell viability or more likely a combination of both of these processes. However, since there was no difference in islet insulin content, insulin mRNA and cell viability between the two strains of mice regardless of culture condition, these cannot explain the changes in insulin release in the DBA/2 mouse following 40 mM glucose exposure for 10 days. This provides further support for our finding that reduction of glucokinase levels may be responsible for the impairment of secretory function following chronic high glucose exposure of DBA/2 but not C57BL/6 islets.

In conclusion, we show that islets from susceptible DBA/2 mice display increased basal insulin secretion and decreased glucose-stimulated secretory function when chronically incubated in high glucose associated with a decrease in glucokinase but not hexokinase levels. Furthermore, treatment with aminoguanidine, which is an inhibitor of the glycation process, was able to reduce basal insulin secretion, increase glucokinase protein levels and prevent this reduction in function in DBA/2 islets. Thus, in our study we demonstrate that hyperglycemia has multiple effects on islets, such as cell viability, reduced insulin content, increased insulin mRNA, which do not vary between mouse strains, yet these strains differ in their susceptibility to β -cell failure. In contrast, islets from a mouse with increased β -cell failure susceptibility have abnormal regulation of glucokinase. How this occurs, or if this is related to subsequent β -cell failure is not clear and warrants further study.

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