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ARTICLE

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The influence of genetic background on the induction of oxidative stress and impaired insulin secretion in mouse islets

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Abstract *Aims/hypothesis:* We determined whether high-glucose-induced beta cell dysfunction is associated with oxidative stress in the DBA/2 mouse, a mouse strain susceptible to islet failure. *Materials and methods:* Glucose- and non-glucose-mediated insulin secretion from the islets of DBA/2 and control C57BL/6 mice was determined following a 48-h exposure to high glucose. Flux via the hexosamine biosynthesis pathway was assessed by determining *O*-glycosylated protein levels. Oxidative stress was determined by measuring hydrogen peroxide levels and the expression of anti-oxidant enzymes. *Results:* Exposure to high glucose levels impaired glucose-stimulated insulin secretion in DBA/2 islets but not C57BL/6 islets, and this was associated with reduced islet insulin content and lower ATP levels than in C57BL/6 islets. Exposure of islets to glucosamine for 48 h mimicked the effects of high glucose on insulin secretion in the DBA/2 islets. High glucose exposure elevated *O*-glycosylated proteins; however, this occurred in islets from both strains, excluding a role for *O*-glycosylation in the impairment of DBA/2 insulin secretion. Additionally, both glucosamine and high glucose caused an increase in hydrogen peroxide in DBA/2 islets but not in C57BL/6 islets, an effect prevented by the antioxidant *N*-acetyl-L-cysteine. Interestingly, while glutathione peroxidase and catalase expression was comparable between the two strains, the antioxidant enzyme manganese superoxide dismutase, which converts superoxide to hydrogen peroxide, was increased in DBA/2 islets, possibly explaining the increase in hydrogen peroxide levels. *Conclusions/interpretation:* Chronic high glucose culture caused an impairment in glucose-stimulated insulin secretion in DBA/2 islets, which have a genetic predisposition to failure, and this may be the result of oxidative stress.

Keywords Anti-oxidants · Glucosamine · Glucose-stimulated insulin secretion · Glucose toxicity · Hexosamine biosynthesis pathway · Oxidative stress

Abbreviations GFAT: glutamine:fructose-6-phosphate amidotransferase-1 · GSIS: glucose stimulated insulin secretion · HBP: hexosamine biosynthesis pathway · H_2O_2: hydrogen peroxide · Mn-SOD: manganese superoxide dismutase · NAC: *N*-acetylcysteine

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Introduction

Two fundamental defects arise in the progression from normal glucose tolerance to type 2 diabetes: a decrease in the ability of tissues to respond to insulin and an inability of beta cells to produce enough insulin to compensate for the insulin-resistant state. Chronic elevations in blood glucose have been shown to induce a number of defects in the islet beta cell. Notably, the term 'glucotoxicity' originated following studies demonstrating the impaired glucose stimulatory effect on insulin secretion following high glucose exposure [1–3]. Despite intense research in this field, the mechanism for the decline in insulin output remains incompletely understood.

A number of studies have implicated 'beta cell exhaustion' as the cause of impaired insulin secretory function [4–6], as well as a reduction in insulin gene transcription factors [7, 8], and glucokinase protein and activity levels [9, 10]. Furthermore, recent evidence suggests that changes in the early steps of insulin receptor signalling may also play a role in hyperglycaemia-induced beta cell dysfunction [11].

One mechanism for hyperglycaemia-induced damage is via the generation of superoxide radicals, which result in oxidative stress [12]. The defence mechanism against this

oxidative stress are antioxidant enzymes like superoxide dismutase and catalase, which convert the superoxide into harmless products. Interestingly, the pancreatic beta cell is particularly sensitive to oxidative stress because of the presence of low levels of antioxidant enzymes [13], thus it can be perceived that hyperglycaemia may cause dysfunction of beta cells via oxidative stress [14]. In fact, this phenomenon has been investigated extensively (reviewed in [15]) and possibly provides the most convincing rationale to explain the reduced secretory capacity of the beta cell following chronic glucose exposure. In support of this, a recent study showed impaired function associated with increased oxidative stress in islets isolated from patients with type 2 diabetes, which was improved when the islets were treated with the anti-oxidant glutathione [16]. On the other hand, what remains in question is how glucose overload generates superoxide. Potential pathways that have been suggested for superoxide production include glycosylation (Schiff reaction) [17], glucose autoxidation [18], glucose metabolism [19, 20] and the hexosamine biosynthesis pathway (HBP) [21]. We have previously shown that regulation of flux through the HBP can concomitantly regulate the secretion of insulin from mouse islets [22].

In the present study, the contribution of the HBP to high glucose-induced changes in islets was investigated in two mouse models with different susceptibility to beta cell failure, the C57BL/6 control strain and the DBA/2 susceptible strain [23, 24]. We show that in response to 2 days of culture in high glucose, only the DBA/2 islets display a reduced insulin response to a glucose stimulus, as well as increased levels of hydrogen peroxide; this suggests the involvement of oxidative stress. These effects are mimicked by incubation with the HBP substrate glucosamine and are reversed following incubation with the antioxidant agent *N*-acetyl-L-cysteine. Therefore, these results suggest a role for high glucose-induced oxidative stress and impaired secretory function, only in genetically predisposed islets.

Materials and methods

Isolation and culture of pancreatic islets

We have previously shown that insulin sensitivity and levels of fasting plasma glucose and insulin were similar in DBA/2 and C57BL/6 mice [25]. Islets were isolated from the pancreas of 7- to 8-week-old male C57BL/6 and DBA/2 mice (Walter and Eliza Hall Institute Animal Research Facility; Kew, VIC, Australia) by collagenase digestion as previously described [22]. Briefly, pancreata were digested by intraductal injection of Collagenase P (0.5 mg/ml) in RPMI-1640 (with L-glutamine) containing 100 U/ml penicillin, 100 μg/ml streptomycin and 11.1 mmol/l glucose. Islets were purified using a Histopaque-1077 density gradient. After the islets were freed from the exocrine tissue, they were hand-picked under a stereomicroscope (Olympus, Tokyo, Japan) and transferred for overnight culture in RPMI-1640 medium with 10% (vol/vol) heat-inactivated fetal calf serum, in a 37°C humidified atmosphere of 95% air: 5% CO_2. All the animal procedures were approved by the Animal Ethics Committee of Austin Health Victoria.

Evaluation of insulin secretion and content

A supraphysiological concentration of glucose (40 mmol/l) was used to culture islets so that a functional defect could be induced in the relatively short incubation period of 48 h. Following overnight culture, batches of islets were transferred to Petri dishes containing RPMI-1640 (with either 11.1 or 40 mmol/l glucose) in the absence or presence of 3 mmol/l glucosamine or *N*-acetylglucosamine and/or 5 mmol/l *N*-acetyl-L-cysteine. Islets were cultured at 37°C for 48 h then preincubated for 90 min in Krebs–Ringer bicarbonate buffer (KRBB) with 2.8 mmol/l glucose. Triplicate batches of five islets each were then transferred to tubes containing 1 ml KRBB supplemented with either 2.8 or 20 mmol/l glucose. Additional stimulation with a cocktail of secretagogues was carried out in some experiments: 0.5 m KRBB was replaced with a secretagogue cocktail containing 0.1 mmol/l 3-isobutyl-1-methylxanthine, 10 mmol/l arginine and 5 μmol/l carbamylcholine chloride (carbachol).

After a 60-min incubation of the islets at 37°C, the tubes were centrifuged at 500 × *g* for 5 min and 0.5 ml supernatant was removed for insulin analysis. The remaining 0.5 ml containing the islets was treated with 0.18 mol/l HCl/95% ethanol, followed by sonication to determine the insulin content.

Insulin levels were determined with a double antibody radioimmunoassay using a rat-specific insulin antibody and rat insulin as a standard (Linco Research; St Charles, MO, USA).

Measurement of islet ATP

ATP was measured as previously described [26] using an ATP assay kit from Sigma (St Louis, MO, USA); 50 μl of each sample or ATP standard was mixed with an equal amount of ATP assay mix, containing luciferase, luciferin, $MgSO_4$, dithiothreitol, EDTA, BSA and tricine buffer salts. The light emitted was measured in a luminometer and is proportional to the ATP present.

Assessment of ^{51}Cr release

Release of ^{51}Cr was used to assess cell viability following exposure to high glucose levels, as previously described [27]. Data were expressed as percentage of ^{51}Cr release in terms of total incorporation (cpm in medium/[cpm in medium + cpm in islets]).

Western blotting analysis of islet glycosylated proteins

Following incubations with test compounds, Western blotting was carried out as previously described [22], with the monoclonal mouse anti-*O*-linked *N*-acetylglucosamine antibody (RL2; Affinity Bioreagents, Golden, CO, USA).

Hydrogen peroxide assay

Hydrogen peroxide levels were determined as previously described [21], using the PeroxiDetect kit (Sigma). Briefly, 20 μl of islet lysate was incubated with aqueous peroxide colour reagent (aqueous solution containing 100 mmol/l sorbitol and 125 μmol/l xylenol orange) and ferrous ammonium sulphate reagent (25 mmol/l ferrous ammonium sulphate in 2.5 mol/l sulphuric acid) for at least 30 min. The H_2O_2 levels were measured in a microtitre plate reader (BioRad Model 680 microplate reader; Bio-Rad, Hercules, CA, USA) by the absorbance at 595 nm. The H_2O_2 levels in the lysates were calculated from the standard curve of nanomole H_2O_2 against optical density at 595 nm.

Real-time PCR

Total RNA was prepared as previously described [28] from islets incubated with 11.1 mmol/l glucose (before high glucose exposure) and 1 μg per reaction was converted to cDNA using the Promega Reverse Transcriptase System kit (Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions. Real-time PCR was carried out in a volume of 10 μl consisting of 1 μl cDNA, 1 × LightCycler enzyme and reaction mix (SYBR Green I dye, *Taq* DNA polymerase, dNTP; Roche Diagnostics Australia, Castle Hill, NSW, Australia), 1.5 mmol/l $MgCl_2$ and 600 nmol of oligonucleotide primers (Proligo Australia, Lismore, NSW, Australia). The oligonucleotide primers were designed with MacVector software (Oxford Molecular, Oxford, UK). All reactions were performed in a LightCycler (Roche), in which samples underwent 40 cycles of PCR with an annealing temperature of 55°C. Standards for each transcript were prepared in a conventional PCR and purified using a High Pure PCR Product Purification Kit (Roche). The following primers were used (forward and reverse): ATGAAGCAGTGGAAGGAGCAGC and CTGTCAAAG TGTGCCATCTCGTC (catalase), ACAGTCCACCGTGTA TGCCTTC and CTCTTCATTCTTGCCATTCTCCTG (glutathione peroxidase), CCACACAGCACTATGTAA AGCGTC and GTTCGGGAAGGTAAAAAAAGCC (haeme oxygenase-1), ATGGGGACAATACACAAGGC TG and CAATGATGGAATGCTCTCCTGAG (Cu/Zn-superoxide dismutase [SOD]), GGGAGATGTTACAACT CAGGTC GC and CCAAAGTCACGCTTGATAGCCTC (Mn-SOD), TCTTCTACACACCCATGTCCC and GGT GCAGCACTGATCTAC (insulin), and TGTGCCAGGG TGGTGACTTTAC and TGGGAACCGTTTGTGTTT GG (cyclophilin). The value obtained for each specific product was expressed relative to the control gene for each sample (ratio of specific product to cyclophilin). These ratios were then expressed as a percentage of the ratio in C57BL/6 islet extracts.

Western blotting and activity assays of anti-oxidant enzymes

The Mn-SOD protein levels were measured in mitochondrial lysates [29] from islets and various tissues both in the normal state and before high glucose exposure using Western blotting and employing a specific polyclonal antibody (cat. no. sc-18503 Santa Cruz Biotechnology, Santa Cruz, CA, USA). Islet catalase and glutathione peroxidase enzyme activity was determined using commercially available kits and following the manufacturer's directions (Cayman Chemical, Ann Arbor, MI, USA). All Western blotting and enzyme activity assays were corrected for the amount of protein in the sample using the Bio-Rad protein assay kit (Bio-Rad).

Statistical analysis

Data are presented as the mean±standard error of the mean (SEM) for the number of experiments indicated. Statistical significance was determined using the two-tailed Student's *t*-test for non-parametric data. A p value <0.05 was considered statistically significant.

Results

Incubation of DBA/2 mouse islets with 11.1 mmol/l glucose for 48 h resulted in a marked increase in glucose-stimulated insulin secretion (GSIS) compared with C57BL/6 islets. We have previously demonstrated this enhanced insulin response in the DBA/2 mouse [30] and found it to be a consequence of increased glucose utilisation. Incubation in 40 mmol/l glucose significantly reduced GSIS from DBA/2 islets, but not from C57BL/6 islets (Fig. 1a). The increase in insulin release (from 2.8 to 20 mmol/l glucose) was ninefold following culture with 11.1 mmol/l glucose but only fourfold following culture with 40 mmol/l glucose for DBA/2 islets. In comparison, in C57BL/6 islets there were fourfold and threefold increases in insulin secretion following culture with 11.1 and 40 mmol/l glucose, respectively. Insulin secretion in response to 20 mmol/l glucose plus a cocktail of secretagogues was unaffected by 40 mmol/l glucose incubation in both strains (Fig. 1b), indicating that the reduction in insulin release in DBA/2 islets was specific to glucose.

To exclude other possible reasons for the reduction in DBA/2 insulin secretion caused by high glucose, ATP levels and cell viability (^{51}Cr-release assay) were analysed in islets incubated in either 11.1 or 40 mmol/l glucose. Table 1 shows that in both strains, basal ATP levels for

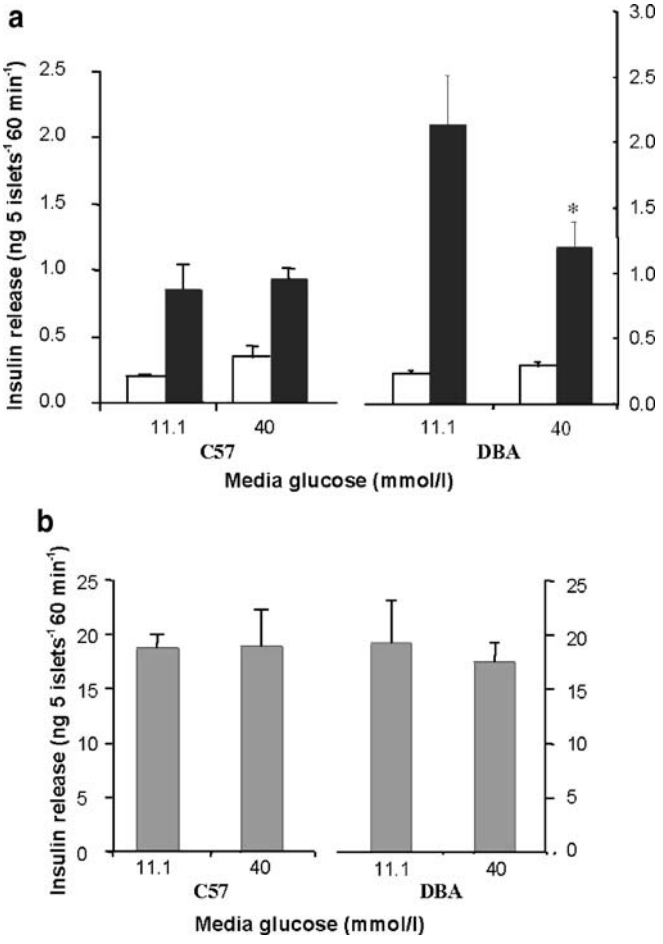

Fig. 1 Insulin secretion following 48 h incubation in either 11.1 or 40 mmol/l glucose. Islets were preincubated in KRBB with 2.8 mmol/l glucose for 90 min and then stimulated for 60 min with either 2.8 (*open bars*) or 20 mmol/l glucose (*closed bars*) (**a**) and 20 mmol/l glucose plus secretagogue cocktail (*shaded bars*) containing 10 mmol/l arginine, 0.1 mmol/l IBMX and 5 μmol/l carbachol (**b**). Values are presented as mean±SEM for five to 13 experiments. *p<0.05 vs 11.1 mmol/l

islets that had been incubated in 40 mmol/l glucose tended to increase compared with those incubated in 11.1 mmol/l glucose. However, when stimulated, the DBA/2 islets incubated in 40 mmol/l glucose had significantly lower ATP levels that those incubated in 11.1 mmol/l glucose. This effect was not seen in C57BL/6 islets. Furthermore, the increase in ATP levels (from 2.8 to 20 mmol/l glucose) was 13.6-fold following culture in 11.1 mmol/l glucose and this increase was considerably less, only 1.7-fold, following 40 mmol/l glucose culture for DBA/2 islets while in C57BL/6 islets the increases in insulin secretion were 2.2-fold and 1.2-fold following 11.1 and 40 mmol/l glucose culture, respectively. ^{51}Cr release (Table 1) was increased in both strains following high glucose exposure, with the levels released from DBA/2 islets being significantly higher than from C57BL/6 islets. Even so, this percentage release (3.45±0.09%) was relatively low and cannot completely explain the impairment in GSIS from the DBA/2 islets.

To investigate the involvement of the HBP on beta cell function in the mouse islets, we studied the effects of its specific substrate glucosamine on insulin secretion. Figure 2 shows that glucosamine mimicked the impairment in GSIS seen in DBA/2 islets following 40 mmol/l glucose incubation, suggesting a role for the HBP in high-glucose-induced beta cell dysfunction. Furthermore, 48 h glucosamine in the presence of 11.1 mmol/l glucose treatment also suppressed GSIS from C57BL/6 islets.

Previously, we have shown that under normal conditions, inhibition of the HBP was associated with modification of insulin secretion by modulating islet protein *O*-glycosylation [22]. That is, when the glutamine:fructose-6-phosphate amidotransferase-1 (GFAT) inhibitor azaserine was used to reduce flux through the HBP and therefore decrease protein *O*-glycosylation, insulin secretion was also reduced. Thus here, we determined whether increased HBP flux, as determined by the level of islet protein *O*-glycosylation, could also be associated with defective insulin secretion. Both C57BL/6 and DBA/2 islets incubated with 40 mmol/l glucose showed an increased level of protein *O*-glycosylation compared with those incubated with 11.1 mmol/l glucose. A representative immunoblot is shown in Fig. 3. This increase was most apparent in the band indicated by the arrow, which has been identified as *O*-*N*-acetylglucosamine transferase [31], the enzyme responsible for the addition of *N*-acetylglucosamine to proteins. Similarly, in both strains of mice co-incubation of 11.1 mmol/l glucose-treated islets with *N*-acetylglucosamine (another substrate for the HBP) also resulted in an increase in *O*-glycosylation compared with incubation in 11.1 mmol/l glucose alone. Therefore the increase in protein *O*-glycosylation following high glucose

Table 1 The effect of 48-h exposure to either 11.1 or 40 mmol/l glucose on ATP levels (n=4) and cell viability (n=3) in C57BL/6 and DBA/2 mouse islets

	C57BL/6		DBA/2	
	11.1 mmol/l	40 mmol/l	11.1 mmol/l	40 mmol/l
ATP (pmol/islet)				
Basal (2.8 mmol/l glucose)	1.12±0.28	2.46±0.86	1.23±0.35	3.59±1.51
Stimulated (20 mmol/l glucose)	2.52±0.83	3.02±0.64	16.30±1.66‡	6.12±1.88*
Fold increase (from 2.8 mmol/l)	2.2	1.2	13.6	1.7
Percentage of ^{51}Cr release	1.76±0.14	2.67±0.08*	1.77±0.09	3.45±0.09*†

*p<0.05 vs 11.1 mmol/l, †p<0.05 vs C57BL/6, ‡p<0.05 vs basal

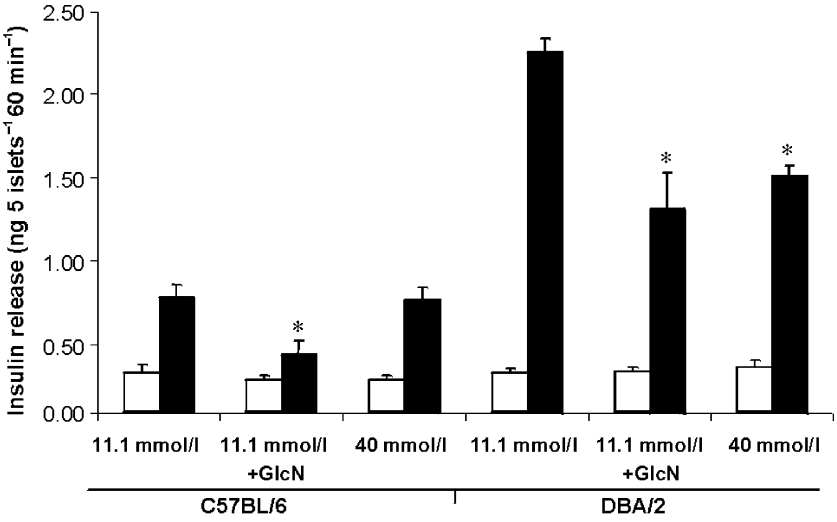

Fig. 2 Insulin secretion following 48 h incubation in either 40 glucose or 11.1 mmol/l glucose in the absence or presence of 3 mmol/l glucosamine (*GlcN*). Islets were preincubated in KRBB with 2.8 mmol/l glucose for 90 min and then stimulated for 60 min with either 2.8 (*open bars*) or 20 mmol/l (*closed bars*) glucose. Values are presented as mean±SEM for six experiments. *p<0.05 vs 11.1 mmol/l

incubation was probably not the cause of reduced GSIS from DBA/2 islets under these conditions.

It has also been shown that a high glucose milieu may cause islet dysfunction via the generation of oxidative stress [12]. Thus an alternative hypothesis could be that 40 mmol/l glucose incubation would induce oxidative stress in the DBA/2 islets, which would then result in impaired GSIS. Hydrogen peroxide levels were measured as an indicator of oxidative stress and found to be elevated in DBA/2 islets incubated with 40 or 11.1 mmol/l glucose plus glucosamine (Fig. 4). Treatment with the antioxidant, *N*-acetyl-L-cysteine (NAC), reversed this increase in H_2O_2 and removed the GSIS defect in DBA/2 islets incubated with 40 mmol/l glucose but it had no effect on insulin release from C57BL/6 islets (Fig. 5).

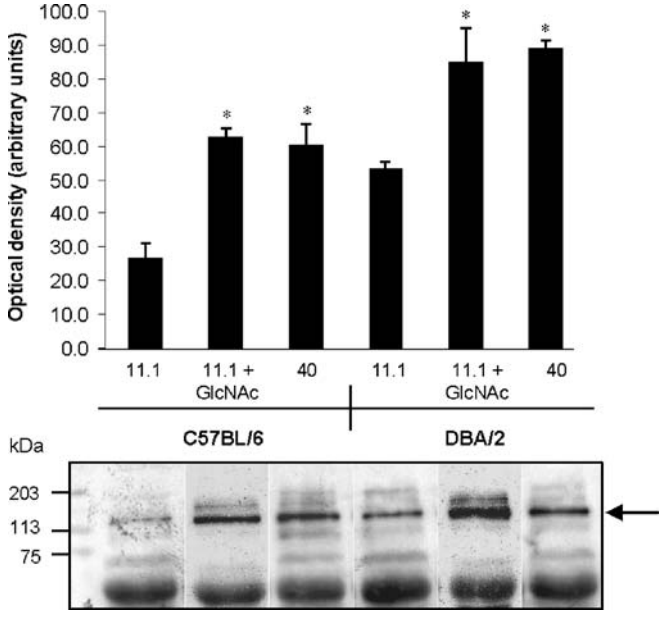

Fig. 3 Islet protein glycosylation following 48 h incubation in either 40 glucose or 11.1 mmol/l glucose in the absence or presence of 3 mmol/l *N*-acetylglucosamine (*GlcNAc*). Western blotting was performed with the RL2 antibody for *O*-linked glycosylation. This immunoblot is a representative of three independent experiments. *p<0.05 vs 11.1 mmol/l

To investigate whether the increase in oxidative stress (as indicated by higher H_2O_2 concentrations) was the result of altered levels of antioxidant enzymes in the DBA/2 strain compared with the C57BL/6 strain, real-time PCR was performed on cDNA prepared from islets before high glucose exposure. Surprisingly, expression of Mn-SOD was elevated and may thus have been responsible for the higher H_2O_2 levels in the DBA/2 islets, while haeme oxygenase-1, glutathione peroxidase, Cu/Zn-SOD and catalase were comparable between the two strains (Fig. 6). Furthermore, high glucose and glucosamine culture did not further increase Mn-SOD in DBA/2 islets (187±10 vs 211±54 vs 187±7, 11.1 vs 40 mmol/l glucose vs glucosamine, respectively, expressed as a percentage of C57BL/6 level). There was also no difference in islet insulin mRNA in the two strains of mice following 11.1 mmol/l glucose incubation.

An increase in Mn-SOD protein levels in DBA/2 compared with C57BL/6 pancreata was also detected using Western blotting (Fig. 7a). Protein levels of Mn-SOD in liver and kidney tissue were comparable between these two strains while there was a modest (25%) increase in heart tissue from DBA/2 mice compared with the C57BL/6 mice (Fig. 7b). Islet activity levels of catalase (12.28±2.42 vs 11.04±1.54 nmol·min^{-1}·mg^{-1} protein; n=3 or n=4, DBA/2 vs C57BL/6) and glutathione peroxidase (0.0124±0.0029 vs 0.0137±0.0023 nmol·min^{-1}·mg^{-1} protein, n=4, DBA/2 vs C57BL/6) were also comparable between DBA/2 and C57BL/6 mice.

To investigate the effect of 40 mmol/l glucose on the levels of intracellular insulin, islet insulin content was measured. As shown in Fig. 8, DBA/2 islets incubated in 40 mmol/l glucose had less insulin content compared with islets incubated in 11.1 mmol/l glucose. Similarly, both C57BL/6 and DBA/2 islets contained less insulin when incubated with 11.1 mmol/l glucose plus glucosamine, compared with 11.1 mmol/l glucose alone. NAC restored insulin content in DBA/2 islets to levels comparable with the 11.1 mmol/l glucose group. Since the secretagogue

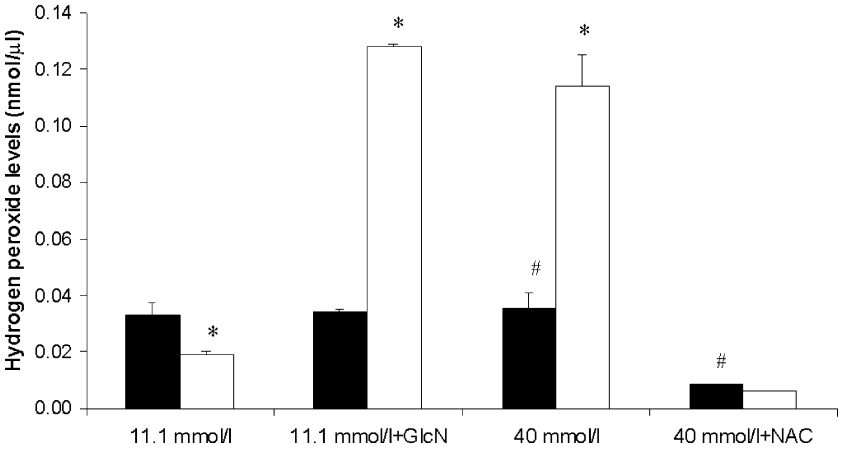

Fig. 4 Hydrogen peroxide levels in C57BL/6 (*closed bars*) and DBA/2 (*open bars*) islets following 48 h incubation in either 11.1 mmol/l glucose with or without 3 mmol/l glucosamine (*GlcN*) or 40 mmol/l glucose with or without 5 mmol/l *N*-acetyl-L-cysteine (*NAC*). Values are presented as mean±SEM for four to six experiments. *p<0.05 vs DBA/2 11.1 mmol/l glucose, #p<0.05 vs DBA/2 40 mmol/l glucose

cocktail was able to evoke an insulin response greater than that of 20 mmol/l glucose alone and comparable with C57BL/6 islets (Fig. 1b), we believe that insulin content alone cannot be seen as the limiting factor for reduced GSIS in DBA/2 islets. Insulin accumulation in the media over the 48-h incubation period was collected and measured to ascertain whether or not beta cell exhaustion was taking place as a result of exposure to 40 mmol/l glucose. Both C57BL/6 and DBA/2 islets secreted significantly more insulin over 48 h when incubated in 40 mmol/l glucose compared with 11.1 mmol/l glucose (DBA/2: 1.38±0.28 ng·islet^{-1}·5 ml^{-1} of 40 mmol/l media vs 0.33±0.06 ng islet^{-1}·5 ml^{-1} of 11.1 mmol/l media; C57BL/6: 1.26±0.24 ng islet^{-1}·5 ml^{-1} of 40 mmol/l media vs 0.16±0.02 ng islet^{-1}·5 ml^{-1} of 11.1 mmol/l media; n=4, p<0.05 40 vs 11.1 mmol/l for both strains). However, the levels in the 40 mmol/l glucose group were comparable between the two strains and therefore cannot explain the reduced insulin content in the DBA/2 islets. Thus, as previously suggested [8], it is likely that 40 mmol/l glucose suppresses insulin biosynthesis in these islets to some extent.

Discussion

The DBA/2 mouse strain displays susceptibility to pancreatic islet failure when stressed with insulin resistance or high glucose [32]. When the *db/db* gene (which encodes for a defective leptin receptor molecule) is expressed on the DBA/2 background, mice are initially hyperinsulinaemic with normoglycaemia but soon develop overt hyperglycaemia as a result of decreased insulin production from a reduced beta cell population [23, 24]. In contrast, *db/db* gene expression on a C57BL/6 genetic background results in marked obesity and insulin resistance, with only mild hyperglycaemia and hyperinsulinaemia as a result of hypertrophy and hyperplasia of beta cells [33]. In the

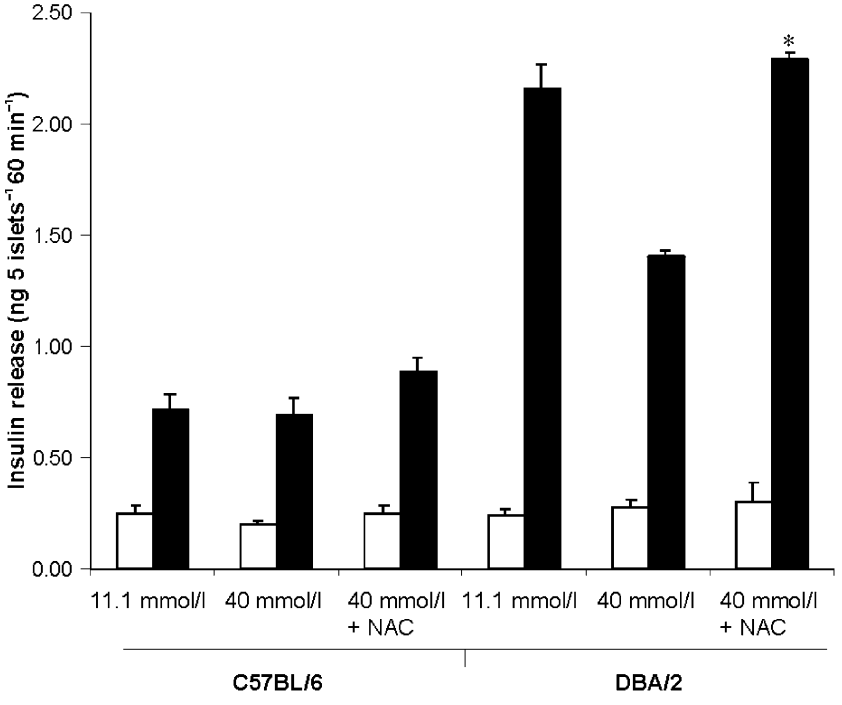

Fig. 5 Glucose-stimulated insulin secretion following 48 h incubation in either 11.1 or 40 mmol/l glucose in the absence or presence of 5 mmol/l *N*-acetyl-L-cysteine (*NAC*). Triplicate batches of five islets each were then transferred to tubes containing 1 ml KRBB supplemented with either 2.8 (*open bars*) or 20 mmol/l (*closed bars*) glucose. Values are presented as mean±SEM for four experiments. *p<0.05 vs 40 mmol/l

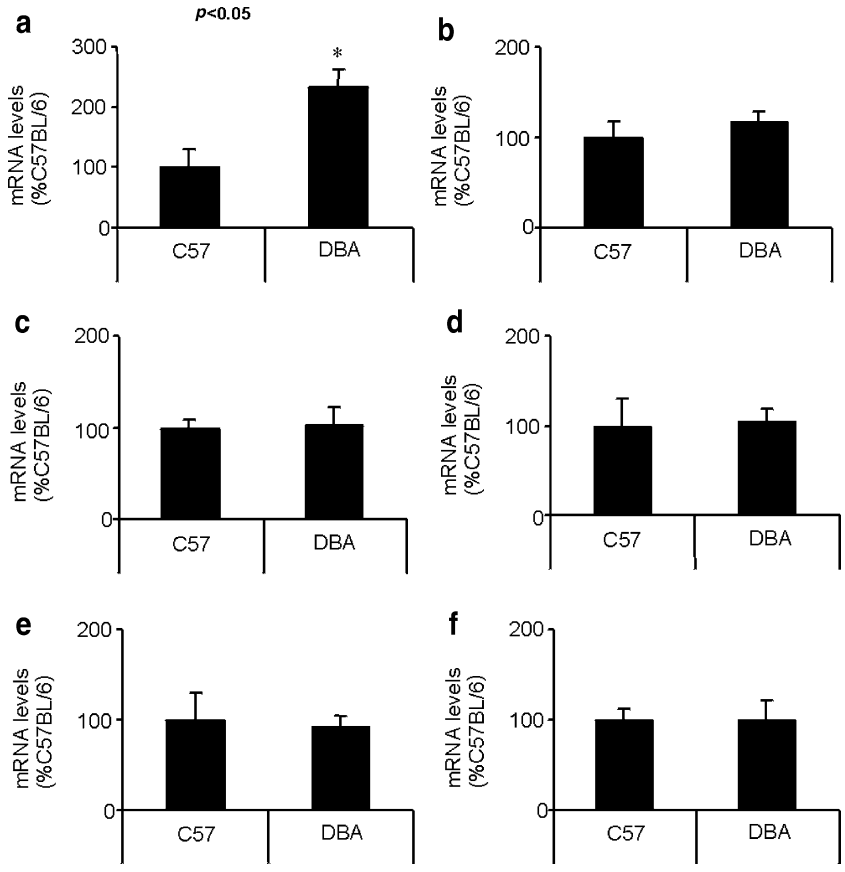

Fig. 6 mRNA levels of Mn-SOD (**a**), glutathione peroxidase (**b**), haeme oxygenase-1 (**c**), Cu/Zn-SOD (**d**), insulin (**e**) and catalase (**f**) as measured by real-time PCR. The value obtained for each specific product was expressed relative to the control gene for each sample (ratio of specific product: cyclophilin). These ratios were then expressed as a percentage of the ratio in C57BL/6 islet extracts. *$p<0.05$ vs C57BL/6

present study, we have compared insulin secretory function in the resilient C57BL/6 strain with that in the susceptible DBA/2 strain following exposure to a high glucose environment and found that only DBA/2 islets display an impairment in GSIS. The data illustrate the influence of genetic background on the development of an insulin secretory defect. The increased levels of H_2O_2 in DBA/2 islets exposed to high glucose concentrations as well as the decreased ATP levels, suggest a role for oxidative stress in the impairment of GSIS.

The HBP has been described as a cellular sensor of energy availability [34, 35], capable of modifying many proteins via *O*-linked glycosylation [36, 37]. We have recently shown that in the normal state, this pathway is involved in regulating the secretion of insulin by altering protein *O*-glycosylation [22]. Other studies have shown that accelerated flux through the HBP, as would be expected in type 2 diabetes, has resulted in beta cell dysfunction [38, 39]. Our present study showed that incubating islets with the HBP substrate glucosamine resulted in reduced GSIS not only in the susceptible DBA/2 mice but also in the C57BL/6 mouse islets. A possible explanation for this finding is that glucosamine is 40 times more potent than glucose in mediating its effects [40] and so an insulin secretion defect was seen in the C57BL/6 islets via mechanisms other than an increase in glycosylated proteins. It is noteworthy that neither 40 mmol/l glucose exposure nor NAC treatment had any effect on C57BL/6 islets, demonstrating that genetic susceptibility plays a significant part in the progression of beta cell dysfunction. This is supported by the inability of NAC to affect insulin secretion in non-diabetic C57BL/KsJ-*misty/misty* mice but the ability normalise GSIS in C57BL/KsJ-*db/db* mice [41].

In the current study, we found that although *O*-glycosylation of islet proteins was elevated following high glucose exposure, it occurred in both strains of mice and, therefore, cannot explain the defect in GSIS from DBA/2 islets. This is supported by a study of rat islets over-expressing GFAT (the rate-limiting enzyme of the HBP), in which GSIS was impaired although not as a result of elevated *O*-glycosylation [21]. In this latter study, the HBP was implicated in oxidative stress-mediated beta cell dysfunction. Rat islets with adenovirus-mediated overexpression of GFAT combined with hyperglycaemia, revealed a reduction in GSIS that was partially restored by treatment with the antioxidant NAC [21]. Similarly, hyperglycaemia in beta cells has been shown to induce oxidative stress [18, 19, 42] and antioxidant drugs have been shown to protect against this effect [41, 43, 44]. The data presented here agree with these studies, showing that H_2O_2 levels are increased in DBA/2 islets exposed to high glucose. Treatment with the antioxidant NAC prevented the rise in H_2O_2 levels while restoring GSIS and insulin content.

The cause of the increase in H_2O_2 levels when DBA/2 islets were exposed to 40 mmol/l glucose was probably an increase in levels of Mn-SOD, a mitochondrial enzyme which forms part of the cell's defence mechanism against

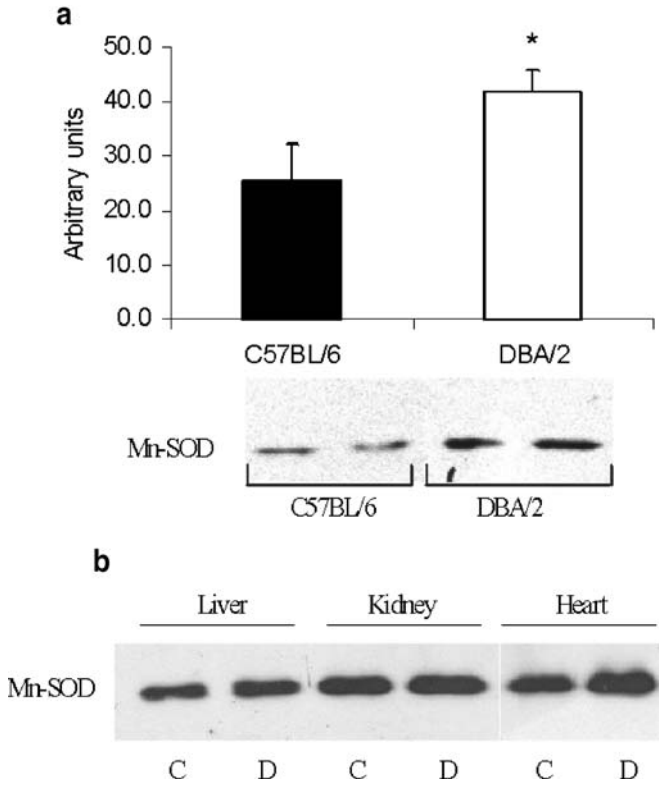

Fig. 7 **a** Mn-SOD protein levels in pancreatic mitochondrial lysates from DBA/2 and C57BL/6 mice. Values are presented as mean± SEM for three experiments. *p<0.05 vs C57BL/6. **b** Mn-SOD protein levels in mitochondrial lysates from liver, kidney and heart tissue from DBA/2 (*lanes D*) and C57BL/6 (*lanes C*) mice

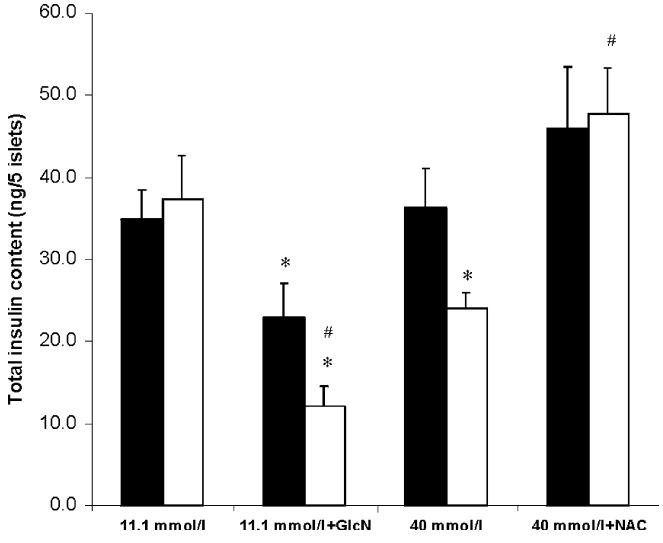

Fig. 8 Islet insulin content in C57BL/6 (*closed bars*) and DBA/2 (*open bars*) islets following 48 h incubation in either 11.1 mmol with or without 3 mmol/l glucosamine (*GlcN*) or 40 mmol/l glucose with or without 5 mmol/l *N*-acetyl-L-cysteine (*NAC*). Values are presented as mean±SEM for four to six experiments. *p<0.05 vs 11.1 mmol/l; #p<0.05 vs 40 mmol/l

oxidative stress. Of interest is the finding that the cytosolic isoenzyme Cu/Zn-SOD was not increased in DBA/2 islets, suggesting that the deleterious effects of high glucose may largely affect mitochondrial function. The role of Mn-SOD is to convert superoxide into H_2O_2 which is then converted to water and molecular oxygen by catalase. Thus the activity of both enzymes is required to safely remove superoxide. However, without a concomitant increase in catalase and glutathione peroxidase levels, an increase in Mn-SOD causes an increase in H_2O_2 in DBA/2 islets exposed to high glucose concentrations and results in reduced GSIS. In support of this, it has been shown that upregulation of Mn-SOD alone does not protect beta cells from glycolysis-generated oxidative damage [45]. In fact, recent studies have shown that overexpression of Mn-SOD resulted in a higher rate of cell death, while catalase overexpression protected against cell death in response to mitochondrially generated superoxide radicals and a cytokine mixture containing IL-1β, TNF-α and IFN-γ in pancreatic (RINm5F) beta cells [46, 47]. Thus, our study and those referred to above support the concept that an increase in Mn-SOD, but not in catalase, will lead to an accumulation of hydrogen peroxide which is deleterious to cell function and viability [48]. It may, therefore, be necessary for a combination of antioxidant enzymes to be elevated for the protection of beta cells against oxidative stress [47, 49].

It has recently been suggested that a mechanism of hyperglycaemia-induced oxidative stress and consequent cell damage occurs via increased glycolysis and glucose oxidation [20, 50]. It is of interest that we have previously shown that DBA/2 islets have increased glucokinase levels and subsequently higher islet glycolytic flux compared to C57BL/6 islets [30]. Furthermore, in the present study we show that two processes that are the result of glucose metabolism, islet ATP and glycosylated protein levels, are also elevated in DBA/2 islets. Thus our data also provide support for the hypothesis that an increase in glycolysis, which results in insulin hypersecretion, may also be responsible for the defects in insulin secretion in the diabetes-prone DBA/2 islets when there is excess substrate availability such as in hyperglycaemia. As a corollary, this hypothesis may also explain why the increased demand placed on the beta cell from insulin resistance or nutrient oversupply results in 'beta cell exhaustion' defects in insulin secretion in individuals with a predisposition to develop type 2 diabetes.

An interpretation of our findings is that the reduction in GSIS in DBA/2 islets following high glucose exposure reflects a return to 'normal' function from a hyper-responsive state. However, given that GSIS was increased back to the pre-glucose exposed state by treatment with an anti-oxidant, we believe that our interpretation of enhanced susceptibility of the DBA/2 islets to glucose toxicity is the more likely explanation of the data. It is difficult to see how oxidative damage could revert cell function to normal.

In conclusion, our data suggest that high glucose impairs GSIS in genetically susceptible islets and that this impairment may be the result of oxidative stress. A better understanding of the underlying genetic factors that contribute to these effects in the DBA/2 mouse may provide clues as to why some individuals develop type 2

diabetes given the necessary environmental stimuli, while others do not.

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