

Glucose-induced beta cell dysfunction in vivo in rats: link between oxidative stress and endoplasmic reticulum stress

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Received: 29 July 2011 / Accepted: 7 December 2011 / Published online: 1 March 2012
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Abstract

Aims/hypothesis Endoplasmic reticulum (ER) stress has been implicated in glucose-induced beta cell dysfunction. However, its causal role has not been established in vivo. Our objective was to determine the causal role of ER stress and its link to oxidative stress in glucose-induced beta cell dysfunction in vivo. **Methods** Healthy Wistar rats were infused i.v. with glucose for 48 h to achieve 20 mmol/l hyperglycaemia with or without the co-infusion of the superoxide dismutase mimetic tempol (TPO), or the chemical chaperones 4-phenylbutyrate (PBA) or tauroursodeoxycholic acid (TUDCA). This was followed by assessment of beta cell function and measurement of ER stress markers and superoxide in islets. **Results** Glucose infusion for 48 h increased mitochondrial superoxide and ER stress markers and impaired beta cell function. Co-infusion of TPO, which we previously found to reduce mitochondrial superoxide and prevent glucose-induced beta cell dysfunction, reduced ER stress markers.

Similar to findings with TPO, co-infusion of PBA, which decreases mitochondrial superoxide, prevented glucose-induced beta cell dysfunction in isolated islets. TUDCA was also effective. Also similar to findings with TPO, PBA prevented beta cell dysfunction during hyperglycaemic clamps in vivo and after hyperglycaemia (15 mmol/l) for 96 h. **Conclusions/interpretation** Here, we causally implicate ER stress in hyperglycaemia-induced beta cell dysfunction in vivo. We show that: (1) there is a positive feedback cycle between oxidative stress and ER stress in glucose-induced beta cell dysfunction, which involves mitochondrial superoxide; and (2) this cycle can be interrupted by superoxide dismutase mimetics as well as chemical chaperones, which are of potential interest to preserve beta cell function in type 2 diabetes.

Keywords Beta cell dysfunction · Endoplasmic reticulum stress · Glucotoxicity · In vivo · Oxidative stress

Electronic supplementary material The online version of this article (doi:10.1007/s00125-012-2474-8) contains peer-reviewed but unedited supplementary material, which is available to authorised users.

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Abbreviations

ATF-6	Activating transcription factor 6
CHOP	c/EBP-homologous protein 10
DI	Disposition index
eIF2 α	Eukaryotic initiation factor-2 α
ER	Endoplasmic reticulum
Ginf	Glucose infusion rate
GRP78	78 kDa glucose-regulated protein
GRP94	94 kDa glucose-regulated protein
HG	High glucose
IRE-1	Inositol-requiring protein-1
ISR	Integrated stress response
KRBH	Krebs–Ringer buffer containing 10 mmol/l HEPES
PBA	4-Phenylbutyrate
PERK	Protein kinase RNA-like endoplasmic reticulum kinase

SAL	Saline (154 mmol/l NaCl) control
s <i>Xbp1</i>	Spliced <i>Xbp1</i>
TPO	Tempol
TUDCA	Tauroursodeoxycholic acid
UPR	Unfolded protein response
u <i>Xbp1</i>	Unspliced <i>Xbp1</i>

Introduction

Type 2 diabetes is characterised by insulin resistance and a defect in insulin secretion, which is inadequate to compensate for insulin resistance (relative insulin deficiency). The progressive failure of beta cells to secrete enough insulin to compensate for insulin resistance leads to hyperglycaemia, which in turn exerts deleterious effects on beta cells [1].

One proposed mechanism of glucose-induced beta cell dysfunction is endoplasmic reticulum (ER) stress [2]. Prolonged *in vitro* exposure of beta cell lines or islets to glucose increased ER stress markers in the majority of studies [3–8], and overproduction of the ER chaperone 78 kDa glucose-regulated protein (GRP78) partially prevented glucose-induced beta cell dysfunction *in vitro* in INS-1 cells [9]. However, it is currently unknown whether ER stress plays a causal role in glucose-induced beta cell dysfunction *in vivo*.

Oxidative stress plays a causal role in glucose-induced beta cell dysfunction both *in vitro* and *in vivo* [1, 10]. Both ER stress [11] and oxidative stress [12] are increased in islets of individuals with type 2 diabetes. Recent studies demonstrate a close inter-relationship between oxidative stress and ER stress in beta cells [13, 14]. Oxidative stress can induce ER stress, and vice versa [2]. However, it is not known whether there is a causal link between oxidative stress and ER stress in the context of glucose-induced beta cell dysfunction.

Previously, we have demonstrated that glucose infusion over 48 h in rats increases reactive oxygen species, and that co-infusion of the superoxide dismutase mimetic tempol (TPO), prevents glucose-induced beta cell dysfunction *in vivo* [10]. The objectives of this study were: (1) to investigate whether ER stress plays a causal role in glucose-induced beta cell dysfunction *in vivo*; and (2) to determine whether there is a link between ER stress and oxidative stress in glucose-induced beta cell dysfunction.

Methods

Animals and surgery

Female Wistar rats (Charles River, Senneville, QC, Canada) received vessel cannulation of the carotid artery (for sampling) and jugular vein (for infusion) [10]. All procedures were approved by the animal care committee at the

University of Toronto. Rats were allowed a minimum of 3 days between surgery and *i.v.* infusions.

Forty-eight hour infusions

Studies with TPO Rats were infused *i.v.* for 48 h with one of: (1) NaCl (154 mmol/l) (SAL); (2) a variable infusion of high glucose (HG; 37.5% wt/vol.) to maintain plasma glucose at 20–22 mmol/l; (3) HG + TPO (Sigma, St Louis, MO, USA; 2.41 $\mu\text{mol kg}^{-1} \text{min}^{-1}$); or (4) TPO as in Tang et al. [10]. TPO was dissolved in NaCl, pH 7.4. Following overnight fasting and immediately after 48 h infusion, islets were isolated to perform the insulin secretion studies described in Tang et al. [10]. In the present manuscript, we report findings for the ER stress markers. Pancreas tissue (collected after the hyperglycaemic clamps described in Tang et al. [10]) was fixed for immunohistochemistry.

Studies with 4-phenylbutyrate Rats were infused *i.v.* for 48 h with one of: (1) SAL; (2) HG as above; (3) HG+4-phenylbutyric acid (PBA [Sigma]; 2.08 $\mu\text{mol kg}^{-1} \text{min}^{-1}$) [15]; or (4) PBA. PBA was dissolved in NaCl, pH 7.4. PBA acts as a chemical chaperone to improve protein folding [16]. Following overnight fasting and immediately after 48 h infusion, we carried out one of the following protocols: (1) insulin secretory studies, superoxide measurements (total and mitochondrial), and ER-stress-marker detection in isolated islets (*ex vivo* studies in islets); or (2) two-step hyperglycaemic clamps followed by pancreas collection for immunohistochemistry.

Studies with tauroursodeoxycholic acid Rats were infused *i.v.* for 48 h with one of: (1) SAL; (2) HG as above; (3) HG + tauroursodeoxycholic acid (TUDCA; Sigma), which also acts as a chemical chaperone [16], at a dose of 2 $\mu\text{mol kg}^{-1} \text{min}^{-1}$ [17]; and (4) TUDCA. TUDCA was dissolved in NaCl, pH 7.4. Islets were isolated for *ex vivo* studies.

Ex vivo studies in islets

Pancreatic islets were isolated with collagenase (Sigma) [10], pre-incubated in Krebs–Ringer buffer containing 10 mmol/l HEPES (KRBH; Wisent, St. Bruno, QC, Canada) and 2.8 mmol/l glucose for 1 h, and then incubated for 2 h at different glucose concentrations. Insulin was measured in the supernatant fraction with Linco's RIA kit (St Charles, MO, USA). The islets used for secretion study were then subjected to acid ethanol extraction [10] for insulin and proinsulin content measurement via RIA and ELISA (ALPCO Diagnostics, Salem, NH, USA), respectively. Total and mitochondrial superoxide were measured as in Tang et al. [10]. Total RNA was extracted from isolated islets using Trizol Reagent (Invitrogen, Burlington, ON, Canada). RNA

was reverse transcribed into single-stranded cDNA, which was used for real-time PCR analysis using the TaqMan Gene Expression System (Applied Biosystems, Foster City, CA, USA) [9]. Primers for *Grp78* (also known as *Hspa5*; Rn01435771_g1), *Chop* (also known as *Ddit3*; Rn00492098_g1), and rat *Ins 2* (Rn01774648g_1) were obtained from Applied Biosystems. Spliced *Xbp1* (*sXbp1*) mRNA was detected as in Zhang et al. [9]. Western blots were performed as in Zhang et al. [9]. The following primary antibodies were used: phospho-eukaryotic initiation factor-2 α (eIF2 α) (Cell Signaling, Danvers, MA, USA; #9721; 1:500); total eIF2 α (Cell Signaling; #9722; 1:1,000); anti-KDEL (amino acid sequence: K, lysine; D, aspartic acid; E, glutamic acid; L, leucine [Stressgen, Brussels, Belgium; SPA-827; 1:1,000]); and γ -tubulin (Sigma; T6557; 1:1,000).

Two-step hyperglycaemic clamp

Insulin secretion was determined by measuring plasma insulin and C-peptide during a two-step (13 and 22 mmol/l) hyperglycaemic clamp in conscious rats after infusion for 48 h with one of: (1) SAL; (2) HG; (3) HG + PBA; and (4) PBA alone (as described above). The clamp details have been previously reported [10] and the evaluation of insulin sensitivity and beta cell function are presented in the electronic supplementary material (ESM Methods).

Immunohistochemistry

At the end of the hyperglycaemic clamp, pancreas was collected [10] and immunostained [18]. The primary antibodies used were mouse monoclonal anti-KDEL (Stressgen; #SPA-827; 1:200), rabbit polyclonal anti-ubiquitin antibody (Dako, Burlington, ON, Canada, Z0458; 1:100) and guinea pig anti-insulin antibody (Dako; Z0564; 1:200). Secondary antibodies used were Oregon Green goat anti-mouse or anti-rabbit IgG (Invitrogen; O11033), Oregon Green goat-anti-rabbit IgG (Invitrogen; O11038) and Cy5-conjugated donkey anti-guinea pig IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA; #706-175-148; 1:200). Images were acquired using a Zeiss LSM510 META laser scanning confocal microscope.

Plasma assays

Linco's RIA kits specific for rat insulin and C-peptide were used.

Ninety-six hour infusions

A set of rats were also infused for 96 h with one of: (1) SAL; (2) a variable infusion of HG (37.5% glucose) to achieve and maintain plasma glucose at 15 mmol/l; (3) HG + PBA; or (4)

PBA alone. The same dose of PBA was used as in the 48 h infusion study. Following the 96 h infusion, islets were isolated for ex vivo studies, which were performed as described above.

INS-1 cells

INS-1 cells were obtained from C. Wollheim (University of Geneva, Switzerland) and cultured as in Asfari et al. [19]. The cells were used within the first few passages upon thawing. INS-1 cells were treated with 1 μ mol/l thapsigargin (Sigma; T9033) as a positive control for the induction of ER stress markers.

Statistical analysis

Data are presented as means \pm SE. One-way non-parametric ANOVA for repeated measurements was used followed by Tukey's *t* test. Calculations were performed using SAS (Cary, NC, USA).

Results

A hyperglycaemic environment for 48 h induces activation of the unfolded protein response in an oxidative-stress-dependent manner

We previously reported that hyperglycaemia for 48 h increased total and mitochondrial superoxide and decreased beta cell function in isolated islets and during hyperglycaemic clamps in vivo, and that this effect was prevented by the antioxidant TPO (a known superoxide mimetic) [10]. To address the link between hyperglycaemia, oxidative stress and ER stress, we measured the ER stress markers.

ER stress activates three sensor proteins, protein kinase RNA-like ER kinase (PERK), inositol-requiring protein-1 (IRE-1) and activating transcription factor 6 (ATF-6), which mediate the unfolded protein response (UPR) [2]. Activated PERK phosphorylates eIF2 α , which attenuates general protein translation. As shown in Fig. 1a,b, hyperglycaemia increased phospho-eIF2 α , and co-infusion of TPO completely prevented this effect. TPO alone did not affect phospho-eIF2 α . As expected, total eIF2 α was not affected by treatment (ESM Fig. 1).

Activated IRE-1 results in splicing of *Xbp1* mRNA, which leads to enhanced *Xbp1* translation. Hyperglycaemia increased *Xbp1* mRNA splicing, and co-infusion of TPO partially prevented this effect. TPO alone mildly increased *sXbp1* mRNA (Fig. 1c,d).

Activation of the UPR leads to increased expression of the pro-apoptotic factor c/EBP-homologous protein 10 (CHOP) and ER chaperones such as GRP78 and 94 kDa glucose-regulated protein (GRP94) [20, 21]. Glucose

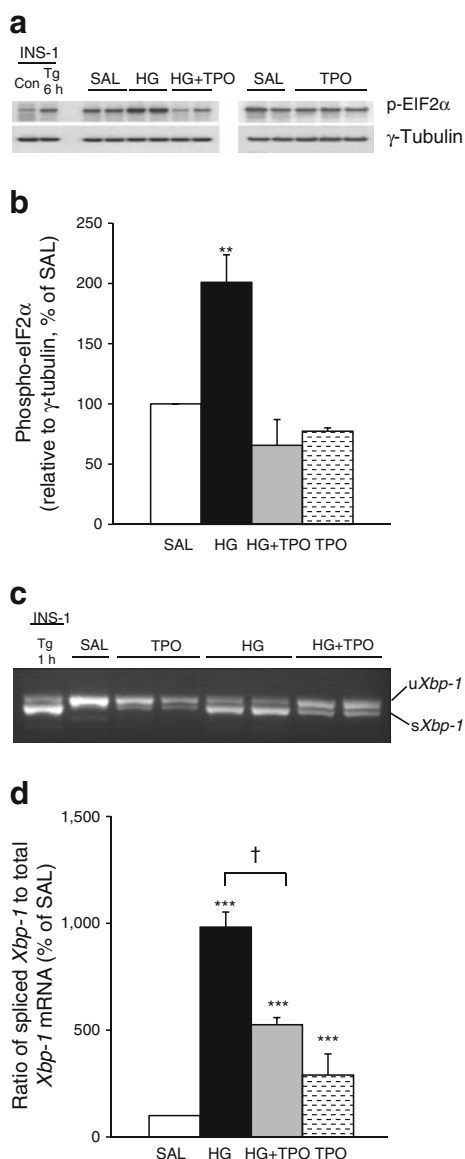


Fig. 1 Hyperglycaemia for 48 h induces activation of the UPR in rat islets in an oxidative-stress-dependent manner. Rats were treated for 48 h, as previously described [10], with: SAL; HG to maintain glycaemia at ~20–22 mmol/l; HG + TPO, 2.41 $\mu\text{mol kg}^{-1} \text{min}^{-1}$; or TPO, 2.41 $\mu\text{mol kg}^{-1} \text{min}^{-1}$. **a** Representative immunoblots of phospho-eIF2 α . Each lane represents islet lysates from one animal. As a positive control, cultured INS-1 cells were treated or not with thapsigargin for 6 h. **b** Western blots for phospho-eIF2 α were quantified and results, normalised by γ -tubulin, are expressed as % of SAL (SAL, $n=5$; HG, $n=7$; HG + TPO, $n=5$; TPO, $n=3$). **c** *Xbp1* mRNA was reverse transcribed and the unspliced (u*Xbp1*; 480 bp) and s*Xbp1* (454 bp) forms of *Xbp1* cDNA were amplified by real-time PCR using primers that flank the intron excised by IRE-1 endoribonuclease activity. Each lane represents islet lysates from one animal. As a positive control, cultured INS-1 cells were treated with thapsigargin for 1 h. **d** The mean of the ratio of s*Xbp1* to total *Xbp1* mRNA (as a % of SAL; SAL, $n=7$; HG, $n=7$; HG + TPO, $n=4$; TPO, $n=4$). Data are means \pm SE. ** $p<0.01$ and *** $p<0.001$ vs SAL; † $p<0.05$ HG + TPO vs HG. Con, control; Tg, thapsigargin

infusion over 48 h increased *Chop* and *Grp78* mRNA. Co-infusion of TPO prevented the high glucose-induced upregulation of *Chop* mRNA, but not *Grp78* mRNA. TPO alone did not affect *Grp78* or *Chop* mRNA (Fig. 2a,b).

Exposure to glucose infusion for 48 h also increased the protein levels of GRP78 and GRP94 (Fig. 2c–e). Co-infusion of TPO did not decrease GRP78 protein and even tended to increase GRP94 protein ($p<0.2$). TPO alone had no effect (Fig. 2c–e). Consistent with the results of the western blot analysis, pancreas sections immunostained with α -KDEL antibody, which recognises GRP78 and GRP94, showed a more intense staining in islets exposed to hyperglycaemia compared with control islets (Fig. 3a,b), while co-infusion of TPO did not decrease α -KDEL staining (Fig. 3c).

The chemical chaperone PBA prevents glucose-induced beta cell dysfunction ex vivo

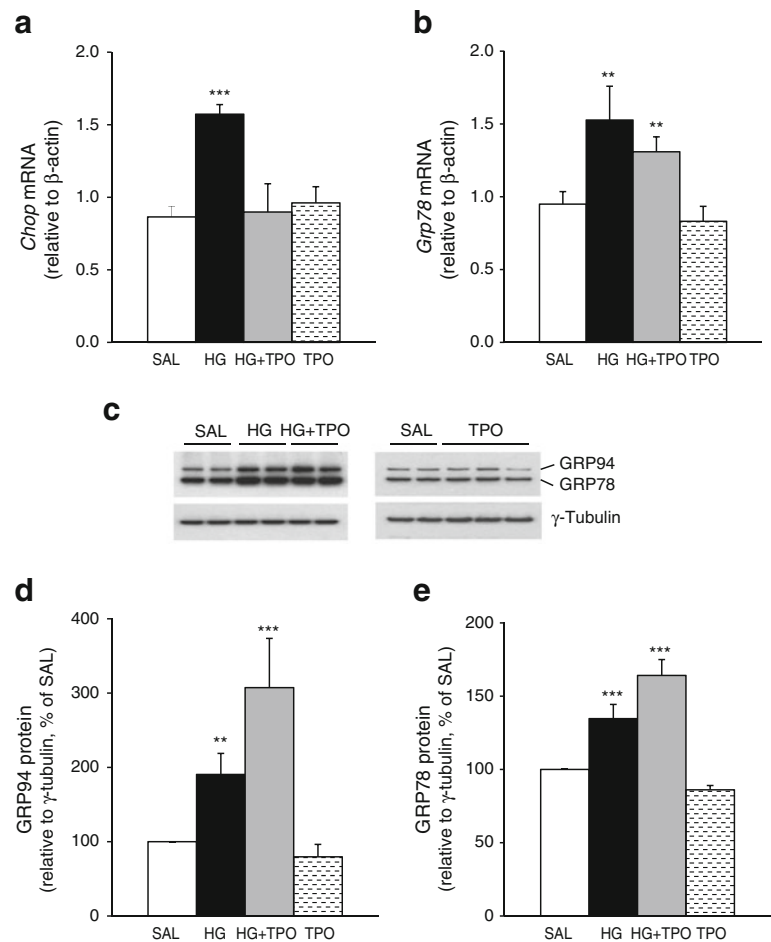
As 48 h hyperglycaemia induced activation of the UPR, we infused PBA, a chemical chaperone, to determine whether it could restore beta cell function in the presence of hyperglycaemia. Following 48 h SAL/HG infusion \pm PBA, islets were isolated and insulin secretion was measured. Insulin secretion at 2.8 mmol/l glucose did not differ between groups (Fig. 4a). Glucose infusion over 48 h increased insulin secretion at 6.5 mmol/l, but impaired insulin secretion at 13 and 22 mmol/l glucose. Co-infusion of PBA with glucose did not significantly affect insulin secretion at 6.5 mmol/l glucose, but completely restored insulin secretion at 13 and 22 mmol/l glucose (Fig. 4a). Glucose infusion over 48 h reduced islet insulin content and this was not improved by co-infusion of PBA (Fig. 4b). Glucose infusion for 48 h did not reduce proinsulin content (indicating that insulin content decreased because of previous hypersecretion), and proinsulin content was not significantly affected by PBA (Fig. 4c).

PBA decreases activation of IRE-1 but not the PERK pathway

To investigate the effect of PBA on activation of UPR, the PERK and IRE-1 pathways were monitored. As shown in Fig. 4d,e, co-infusion of PBA with glucose did not decrease glucose-induced phospho-eIF2 α . However, PBA co-infusion reduced s*Xbp1* mRNA (Fig. 4f, g), suggesting that PBA decreases activation of IRE-1. Unexpectedly, PBA alone increased phospho-eIF2 α and slightly elevated s*Xbp1* mRNA.

Co-infusion of PBA partially decreased *Chop* and *Grp78* mRNA induced by 48 h glucose infusion (Fig. 5a,b). Similar to TPO, co-infusion of PBA did not decrease GRP78 protein, and significantly increased GRP94 compared with

Fig. 2 Effects of hyperglycaemia and TPO on markers downstream of UPR activation. Total RNA was extracted from islets isolated from rats treated as described in the legend of Fig. 1. Real-time RT-PCR was used to analyse the relative mRNA levels of *Chop* (a) and *Grp78* (b). Results are expressed as target gene relative to β -actin ($n=4$ rats per group). c Representative immunoblots with the α -KDEL antibody that recognises GRP78 and GRP94 as indicated. Each lane represents islet lysates from one animal. Western blots for GRP94 (d) and GRP78 (e) were quantified and results are expressed as % of SAL normalised by γ -tubulin (SAL, $n=9$; HG, $n=10$; HG + TPO, $n=5$; TPO, $n=3$). Data are means \pm SE. ** $p<0.01$ and *** $p<0.001$ vs SAL



glucose infusion alone (Fig. 5c–e). PBA alone slightly increased *Chop* and *Grp78* mRNA, and GRP78 and GRP94 protein levels compared with control. Consistent with our western blot data, immunostaining for α -KDEL showed greater staining in the glucose-infused groups compared with control, irrespective of PBA (Fig. 3b,d).

PBA reduces total and mitochondrial superoxide

Glucose infusion for 48 h elevated total and mitochondrial islet superoxide, as previously reported [10]. Co-infusion of PBA with glucose significantly lowered both total and mitochondrial superoxide. PBA alone reduced total superoxide, but did not affect mitochondrial superoxide (Fig. 6).

TUDCA, another chemical chaperone, also prevents glucose-induced beta cell dysfunction ex vivo

As PBA may have non-specific effects, including the inhibition of histone deacetylation [15], we performed experiments with TUDCA, another chemical chaperone. Similar to findings with PBA, co-infusion of TUDCA—which decreased hyperglycaemia-induced *Xbp1* mRNA splicing and *Chop* mRNA (ESM Fig. 2a,b)—did not alter the effect of

hyperglycaemia on insulin secretion at 6.5 mmol/l glucose, but completely restored insulin secretion at 13 and 22 mmol/l glucose (Fig. 7a). Also similar to PBA, TUDCA did not improve islet insulin or proinsulin content (Fig. 7b, c).

PBA prevents glucose-induced beta cell dysfunction in vivo

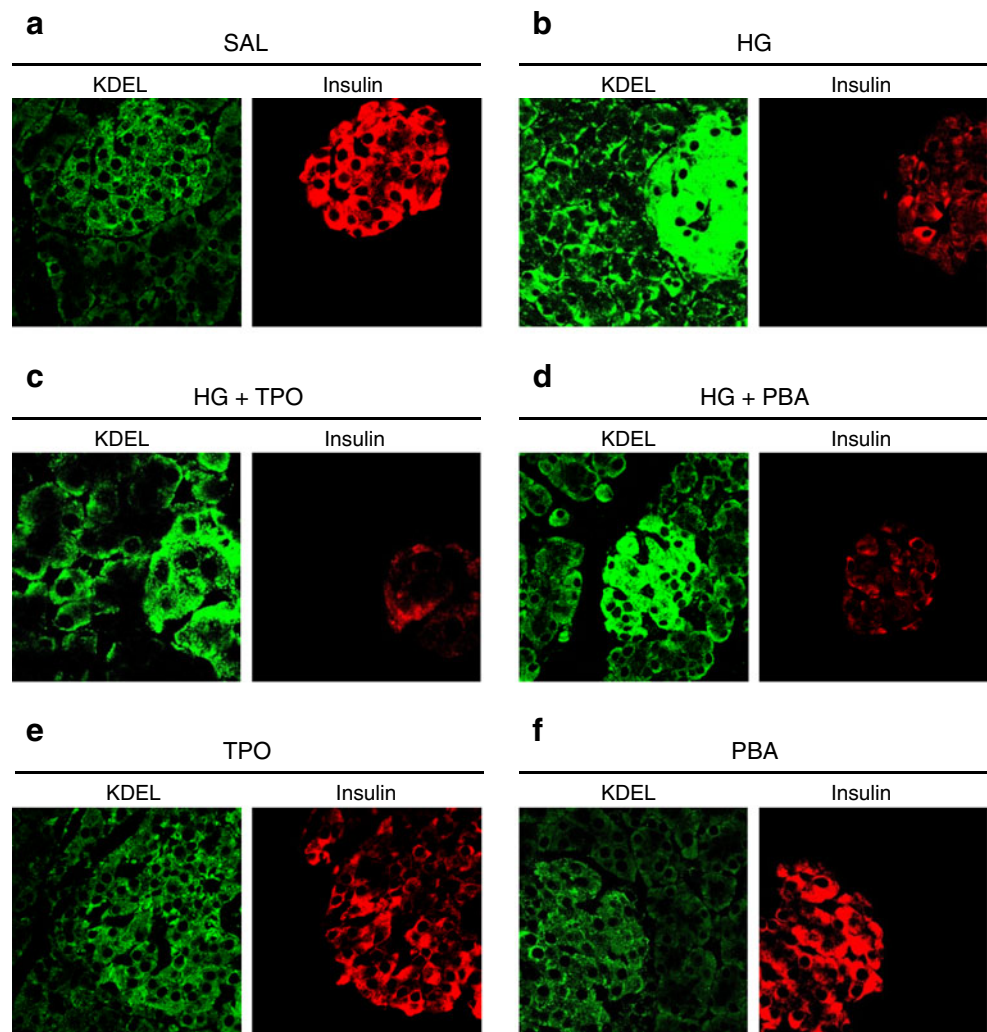
To investigate whether PBA is effective in preventing glucose-induced beta cell dysfunction in vivo, a two-step hyperglycaemic clamp was performed, as with our studies with TPO [10]. Basal plasma glucose before the clamp was lower in the groups previously infused with glucose (HG and HG + PBA). Plasma glucose was elevated to 13 mmol/l and then to 22 mmol/l, with no difference between groups (Fig. 8a).

In the HG group, a lower glucose infusion rate (Ginf) was necessary to clamp glucose at 22 mmol/l, indicating that the circulating insulin was inadequate to compensate for insulin resistance. Co-infusion of PBA completely normalised Ginf (Fig. 8b).

Basal insulin and C-peptide levels were higher in the groups previously infused with glucose (HG and HG + PBA) (Fig. 8c,d). During the hyperglycaemic clamps, in response to increasing glucose levels, plasma insulin and

Fig. 3 Effects of TPO and PBA on GRP78 and GRP94 levels in pancreatic tissue sections at the end of 48 h glucose infusion. Pancreatic tissue sections obtained from rats treated as described in the legends of Figs 1 and 4.

Sections were co-stained with α -KDEL (green) and α -insulin (red) antibodies and images were obtained using a laser scanning confocal microscope. **a** SAL; **b** HG; **c** HG + TPO; **d** HG + PBA; **e** TPO; and **f** PBA. Increased staining with the α -KDEL antibody in HG pancreas was also evident in the surrounding acinar cells, perhaps indicative of some ER stress occurring in these cells. This is consistent with our previous findings [18]



C-peptide levels rose as expected. The HG group had elevated plasma insulin at both 13 mmol/l and 22 mmol/l glucose, and C-peptide at 13 mmol/l glucose. Compared with the HG group, the HG + PBA group tended to show lower plasma insulin, but higher C-peptide levels, at 22 mmol/l (Fig. 8c,d).

The C-peptide/insulin ratio (index of insulin clearance) was lower with HG than with SAL at 13 mmol/l ($p < 0.05$ vs SAL; SAL = 3.66 ± 0.26 vs HG = 2.11 ± 0.09) and 22 mmol/l glucose ($p < 0.05$ vs SAL; SAL = 3.08 ± 0.20 vs HG = 2.10 ± 0.09). The decrease in the C-peptide/insulin ratio was partially prevented by the co-infusion of PBA (HG+PBA = 2.56 ± 0.17 at 13 mmol/l glucose; 2.51 ± 0.21 ; NS vs SAL or HG at both glucose levels). Sensitivity index (M/I index: glucose metabolism (M), which is represented by G_{inf} , divided by plasma insulin (I); see [ESM Methods](#) for calculation) was decreased in HG compared with SAL at both 13 and 22 mmol/l glucose. Co-infusion of PBA tended to prevent this decrease (Fig. 8e).

Although insulin and C-peptide levels were elevated in HG, the disposition index (DI), which takes into account the ability of the beta cell to compensate for insulin resistance

(see [ESM Methods](#) for a description of DI), was lower with HG than with SAL. PBA completely normalised DI during both steps of the clamp (Fig. 8f).

PBA prevents beta cell dysfunction in a more chronic, milder model of hyperglycaemia

As in our previous study with TPO, we also investigated the effect of PBA in a less extreme, more prolonged model of hyperglycaemia. Previous hyperglycaemia at 15 mmol/l for 96 h increased insulin secretion at 2.8 and 6.5 mmol/l glucose, but impaired insulin secretion at 13 and 22 mmol/l glucose. Similar to the effect of TPO seen previously [10], PBA did not prevent the increase in insulin secretion at 2.8 and 6.5 mmol/l induced by hyperglycaemia, but prevented the impairment in insulin secretion at 13 and 22 mmol/l glucose (Fig. 9a). Islet insulin content was reduced by 96 h of hyperglycaemia. This was partially prevented by PBA co-infusion (Fig. 9b). Hyperglycaemia for 96 h did not decrease islet proinsulin content. However, co-infusion of PBA significantly elevated proinsulin content (Fig. 9c). Phospho-eIF2 α levels

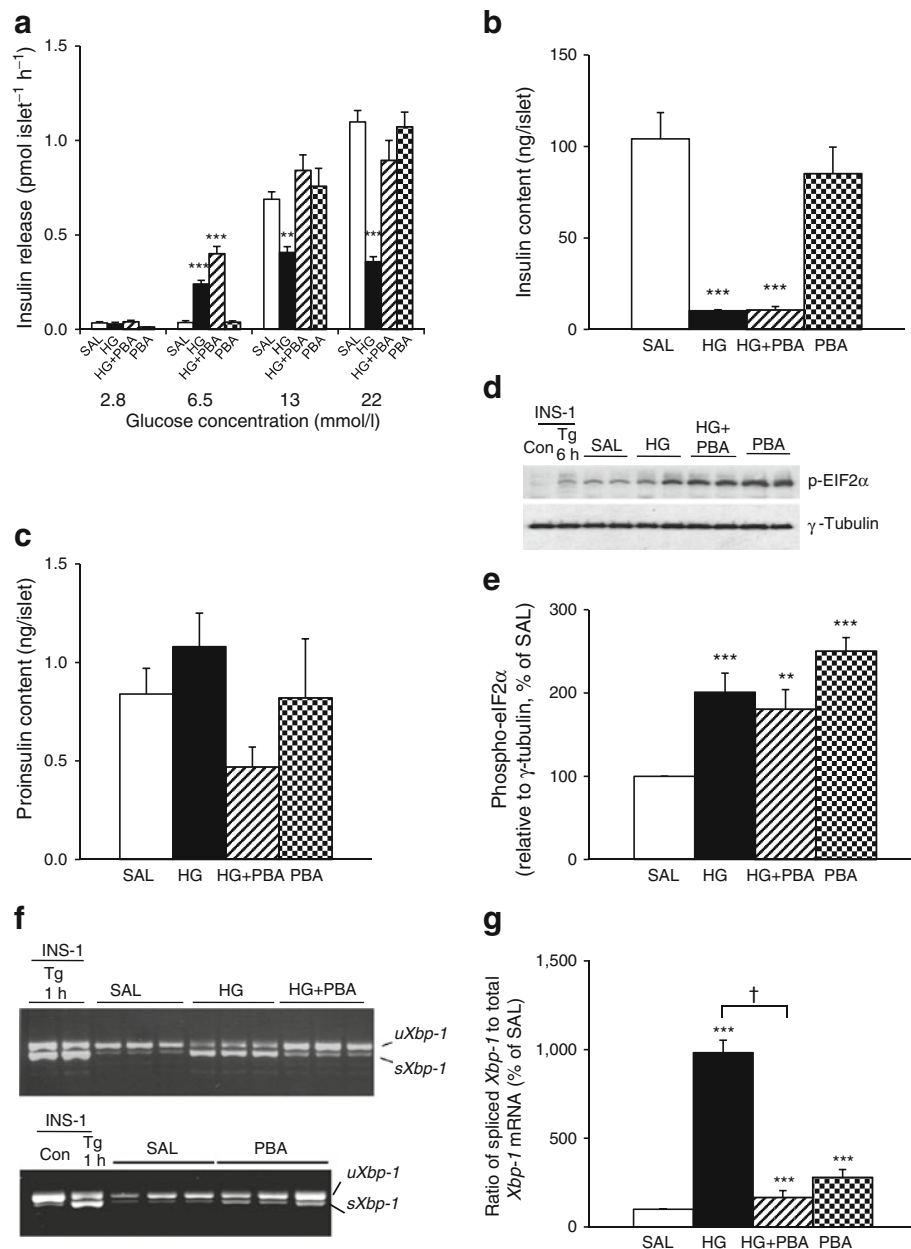


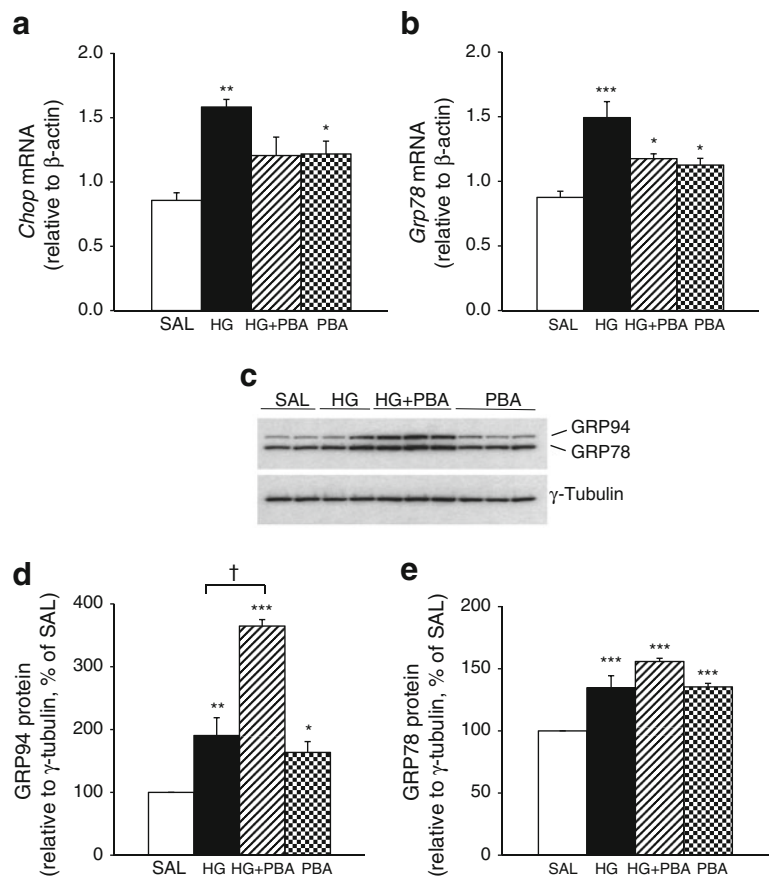
Fig. 4 PBA prevents glucose-induced beta cell dysfunction ex vivo, and decreases activation of the IRE-1 pathway. Rats were treated for 48 h with: SAL; HG; HG + PBA, $2.08 \mu\text{mol}/\text{kg}^{-1} \text{min}^{-1}$; or PBA, $2.08 \mu\text{mol}/\text{kg}^{-1} \text{min}^{-1}$. Following 48 h infusion, islets were isolated and pre-incubated in KRBH containing 2.8 mmol/l glucose for 1 h at 37°C . Thereafter, five islets of approximately the same size were transferred in triplicate to vials containing fresh medium plus glucose at 2.8, 6.5, 13 and 22 mmol/l. Islets were incubated for 2 h at 37°C , and the insulin released was measured in the supernatant fraction (SAL, $n=12$; HG, $n=12$; HG + PBA, $n=10$; PBA, $n=6$) (a). The islet pellets were subjected to acid-ethanol extraction for measurement of insulin content using RIA (SAL, $n=10$; HG, $n=11$; HG + PBA, $n=8$; PBA, $n=6$) (b) and proinsulin content using ELISA (SAL, $n=3$; HG, $n=3$; HG + PBA, $n=4$; PBA, $n=4$) (c). d A representative immunoblot of

phospho-eIF2 α . Each lane represents islet lysates from one animal. As a positive control, cultured INS-1 cells were treated or not treated with thapsigargin for 6 h. Western blots for phospho-eIF2 α were quantified and the results normalised by γ -tubulin are expressed as % of SAL (e) (SAL, $n=5$; HG, $n=7$; HG + PBA, $n=5$; PBA, $n=5$). f *Xbp1* mRNA was reverse transcribed and *uXbp1* and *sXbp1* were amplified by real-time PCR using primers that flank the intron excised by IRE-1 endonuclease activity. Each lane represents islet lysates from one animal. As a positive control, cultured INS-1 cells were treated with thapsigargin for 1 h and two independent samples are shown. g The mean of the ratio of *sXbp1* to total *Xbp1* mRNA (as a % of SAL; SAL, $n=7$; HG, $n=7$; HG + PBA, $n=3$; PBA, $n=3$). Data are means \pm SE. $**p<0.01$ and $***p<0.001$ vs SAL; $^\dagger p<0.05$ HG + PBA vs HG. Con, control; Tg, thapsigargin

tended to be lower with hyperglycaemia and PBA alone. In the HG + PBA group, the decrease was significant vs control,

but not vs HG alone (Fig. 9d,e). Hyperglycaemia increased *Xbp1* mRNA splicing and PBA co-infusion decreased *sXbp1*

Fig. 5 Effects of hyperglycaemia and PBA on markers downstream of UPR activation. Total RNA was isolated from islets isolated from rats treated as described in the legend of Fig. 4. Real-time RT-PCR was used to analyse the relative mRNA levels of *Chop* (a) and *Grp78* (b). Results are expressed as target gene relative to β -actin ($n=4$ rats per group). c A representative immunoblot with the α -KDEL antibody that recognises GRP78 and GRP94. Each lane represents islet lysates from one animal. Western blots for GRP94 (d) and GRP78 (e) were quantified and results are expressed as % of SAL normalised by γ -tubulin (SAL, $n=9$; HG, $n=10$; HG + PBA, $n=5$; PBA, $n=5$). Data are means \pm SE. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ vs SAL; † $p<0.01$ HG + PBA vs HG



mRNA levels below those in the control (Fig. 9f,g). PBA alone decreased *sXbp1* mRNA, suggesting that, chronically, islets adapt to PBA so that the elevation of some ER stress markers observed at 48 h no longer persists. As *Xbp1* mRNA splicing in SAL was greater at 96 than 48 h (Fig. 9f vs Fig. 4f), another possibility is that PBA protected against this increase.

Discussion

We here show that in vivo islet exposure to HG for 48 h increased activation of the UPR. Co-infusion of TPO decreased markers of UPR activation induced by glucose (phospho-eIF2 α , *sXbp1* mRNA and *Chop* mRNA). Our previous study shows that co-infusion of TPO with glucose decreases total and mitochondrial superoxide in islets [10]. A role of antioxidants in reducing ER stress to improve protein secretion has been documented in other cell types [22]. Recent studies demonstrate that the ER and mitochondria interact both spatially and functionally [23]. We hypothesise that an increase in mitochondrial superoxide production in the electron transport chain during hyperglycaemia may leak into the ER. This may impair protein folding [24] and deplete ER calcium by inhibiting sarcoplasmic reticulum ATPase [25, 26], leading to ER stress.

Thus, it is possible that TPO mitigates ER stress via reducing mitochondrial superoxide.

The observation that TPO prevents the glucose-induced increase in ER stress markers supports the notion that ER stress lies downstream of oxidative stress. However, there is also evidence that ER stress can induce oxidative stress [13, 14, 27]. In the present study, PBA reduced total and mitochondrial superoxide levels in islets, suggesting that ER stress may impair beta cell function by inducing oxidative stress. ER stress can increase superoxide via Ca²⁺ leakage from the ER, and uptake into mitochondria, which can subsequently cause disruption of the electron transport chain [28].

In the 48 h infusion model, co-infusion of PBA with glucose decreased *Xbp1* mRNA splicing, and *Chop* mRNA, similar to TPO. Neither PBA nor TPO decreased GRP78 and GRP94 protein levels. GRP94 was actually increased by PBA and tended to be increased with TPO. The reason for this effect of PBA/TPO is unclear. However, it is possible that co-infusion of PBA/TPO results in a milder degree of ER stress than glucose infusion alone, which allows the beta cell to increase the production of chaperones [29]. Interestingly, TPO, but not PBA, prevented the glucose-induced increase in phospho-eIF2 α . Also interestingly, after 96 h of 15 mmol/l HG, a milder model of hyperglycaemia,

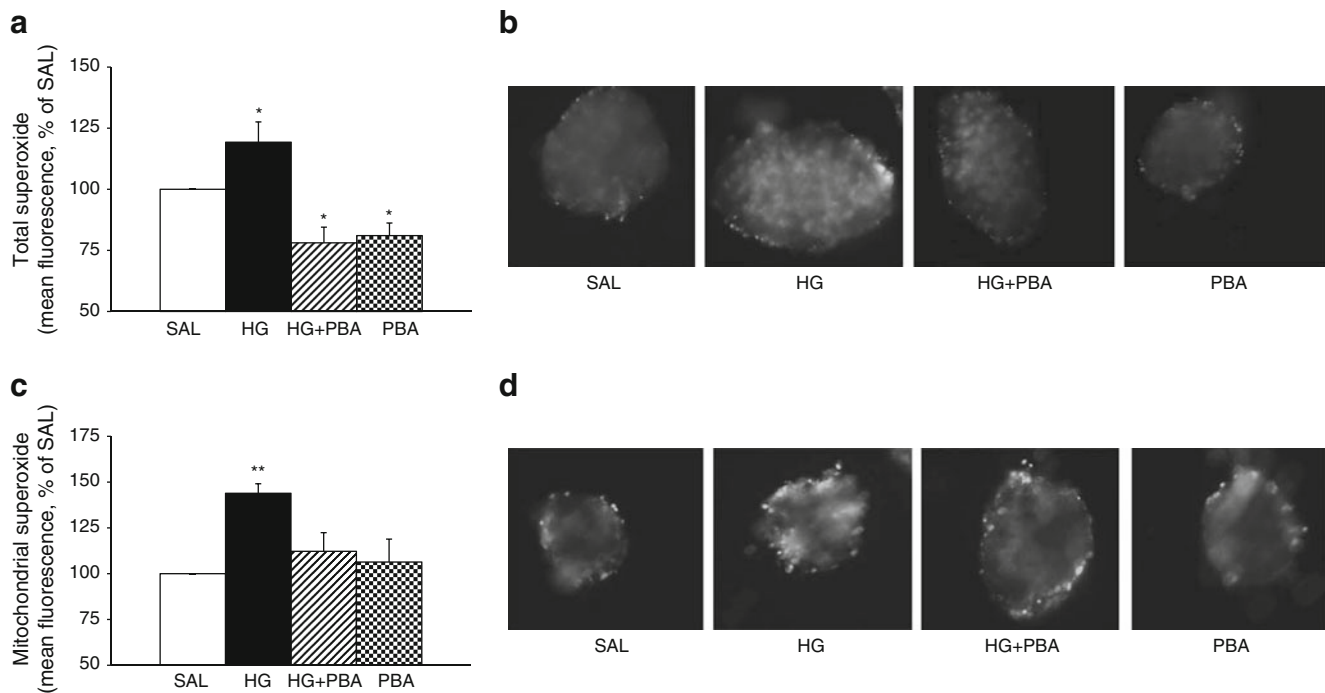


Fig. 6 Effects of hyperglycaemia and PBA on total and mitochondrial superoxide levels in freshly isolated islets. Animals were treated as described in the legend of Fig. 4 and islets were isolated. Following 1 h pre-incubation at 37°C in KRBH buffer containing either 2.8 mmol/l or 20 mmol/l glucose (for glucose-infused groups), islets were incubated with hydroethidine (3 μ mol/l) (**a,b**) or MitoSOX (5 μ mol/l) (**c,d**), in KRBH buffer containing 2.8 mmol/l glucose for 15 min at 37°C. After

washing with KRBH, islet fluorescence was measured at 550 nm excitation and 660 nm emission using an Olympus fluorescence BX51W1 microscope. Approximately 20 islets were measured in each rat (SAL, $n=9$; HG, $n=9$; HG + PBA, $n=8$; PBA, $n=7$). Data are expressed as % of SAL \pm SE (an SAL control rat was studied on each experiment day). * $p<0.05$ and ** $p<0.01$ vs SAL. **b** and **d** show representative images with hydroethidine and MitoSOX, respectively

phospho-eIF2 α was not increased but tended to be decreased by HG, as previously found (glucose induces dephosphorylation of eIF2 α [30]). eIF2 α is not only phosphorylated by PERK, but also by other known kinases activated, for example, under conditions of viral infection, amino acid deprivation or haem deficiency [4, 31, 32]. Although these conditions have little or no relevance to our model, additional, as yet unidentified, kinases may phosphorylate eIF2 α in response to oxidative stress (perhaps independently of ER stress) as part of the integrated stress response (ISR) [32]; thus, the increase in phospho-eIF2 α and *Chop* mRNA may be due to the ISR rather than the UPR as a result of the oxidative stress associated with hyperglycaemia. However, the robust activation of *Xbp1* splicing (reflective of IRE-1 activation by the UPR) indicates that hyperglycaemia does induce ER stress in pancreatic beta cells. Furthermore, 96 h hyperglycaemia at 15 mmol/l did not affect the ISR, based on our eIF2 α results, but induced *Xbp1* splicing. Findings similar to ours, which show a differential ISR response to different glucose concentrations but a progressive induction of the UPR by raising the glucose concentrations, have been reported previously [4].

Our conclusions regarding the involvement of ER stress in glucose-induced beta cell dysfunction are strengthened by the results obtained with TUDCA, another chemical chaperone, which also restored beta cell function.

One limitation of the 48 h HG in vivo model is that it is very extreme and short term, and may not represent glucotoxicity in humans. However, glucose-induced beta cell dysfunction does occur in humans exposed to hyperglycaemia (~13 mmol/l) for 68 h [33]. Experimental hyperglycaemia due to prolonged glucose infusion is not well tolerated by humans [34], therefore mechanistic studies need to be performed in animals. Previously, we have shown that TPO prevents glucose-induced beta cell dysfunction following 96 h hyperglycaemia (15 mmol/l) in rats [10]. In this present study, we show that PBA is also effective in this model, suggesting that ER stress does play a role in a milder and more chronic model of glucose-induced beta cell dysfunction.

ER stress can impair beta cell function by a number of mechanisms. ER stress can activate PERK to decrease insulin biosynthesis at the level of translation via eIF2 α phosphorylation. However, we do not believe this is likely in our model because at 48 h, PBA prevented beta cell dysfunction without preventing the HG-induced increase in phospho-eIF2 α , and because at 96 h, hyperglycaemia did not increase phospho-eIF2 α levels. Another possibility is that activation of the UPR leads to activation of c-jun N-terminal kinase (JNK) [35], which can impair insulin gene transcription via pancreatic duodenal homeobox 1 (PDX-1) nuclear exclusion [36] resulting in impaired insulin biosynthesis, and/or

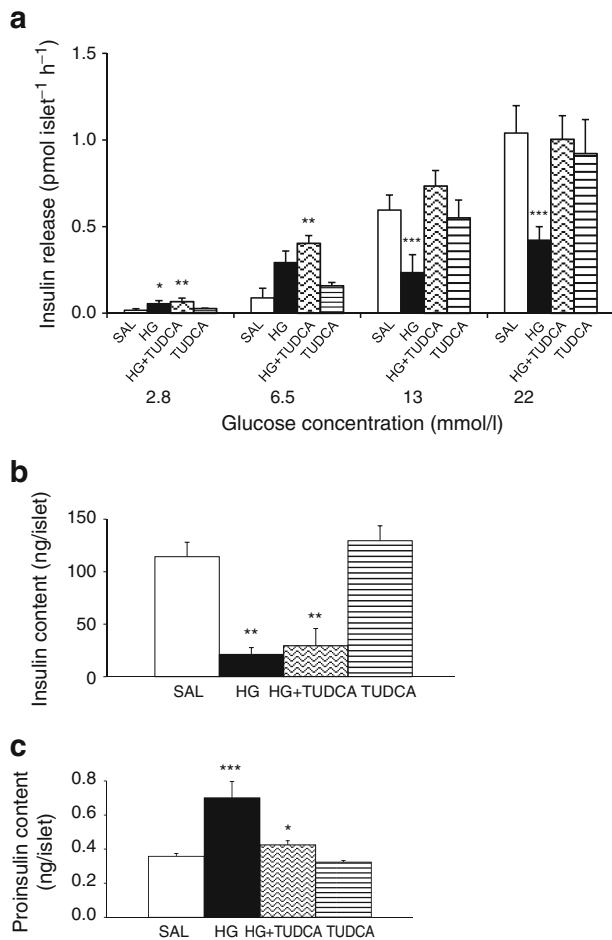


Fig. 7 TUDCA prevents glucose-induced beta cell dysfunction ex vivo. Rats were treated for 48 h with: SAL; HG; HG + TUDCA, $2 \mu\text{mol kg}^{-1} \text{min}^{-1}$; or TUDCA, $2 \mu\text{mol kg}^{-1} \text{min}^{-1}$. Following 48 h infusion, islets were isolated and pre-incubated in KRHB containing 2.8 mmol/l glucose for 1 h at 37°C. Thereafter, five islets of approximately the same size were transferred in triplicate to vials containing fresh medium plus glucose at the following concentrations: 2.8, 6.5, 13 and 22 mmol/l. Islets were incubated for 2 h at 37°C, and the insulin released was measured in the supernatant fraction (SAL, $n=7$; HG, $n=7$; HG + TUDCA, $n=6$; TUDCA, $n=4$) (a). The islet pellets were subjected to acid-ethanol extraction for measurement of insulin content using RIA (SAL, $n=5$; HG, $n=6$; HG + TUDCA, $n=5$; TUDCA, $n=4$) (b) and proinsulin content using ELISA (SAL, $n=4$; HG, $n=4$; HG + TUDCA, $n=3$; TUDCA, $n=3$) (c). Data are means \pm SE. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ vs SAL

can directly impair insulin biosynthesis at the level of translation [37]. Indeed, we found that 48 h hyperglycaemia tended to reduce preproinsulin mRNA which was restored by TUDCA (ESM Fig. 2c) but that neither PBA nor TUDCA attenuated the hyperglycaemia-induced marked impairment in insulin content, suggesting little effect of chemical chaperones on insulin biosynthesis at 48 h. In addition to the impairment of insulin biosynthesis, ER stress can induce mitochondrial superoxide production, which can impair glucose oxidation [38] and activate uncoupling protein 2 (UCP2) [39] to decrease ATP production, and hence

insulin granule exocytosis. Thus, it is likely that with extreme short-term hyperglycaemia (48 h model), alleviation of ER stress mainly improves beta cell function at the level of glucose-induced exocytosis as a result of reduced mitochondrial superoxide and improved mitochondrial function rather than at the level of insulin biosynthesis. Interestingly, following 96 h infusion, PBA partially prevented the decrease in insulin content, and increased islet proinsulin content. Thus, with milder, more prolonged hyperglycaemia (96 h), alleviation of ER stress may eventually improve insulin biosynthesis, perhaps as a result of transcriptional effects that will have to be further addressed in future studies.

Co-infusion of PBA also prevented glucose-induced beta cell dysfunction in vivo, as assessed by the DI (an in vivo measure of beta cell function, including the beta cell ability to compensate for insulin resistance). PBA tended to improve insulin sensitivity. Insulin sensitivity during a hyperglycaemic clamp mainly reflects peripheral insulin sensitivity because glucose production is totally suppressed by glucose and high insulin. It has been previously reported that PBA reduces obesity-induced ER stress and consequently improves insulin sensitivity in the liver [40]. We have shown that PBA partially prevents the glucose-induced decrease in insulin clearance (as indicated by the C-peptide to insulin ratio). Insulin clearance is mainly a liver function, and is generally proportional to hepatic insulin sensitivity. Thus, PBA may prevent glucose-induced hepatic insulin resistance. As PBA can increase insulin clearance, it cannot be excluded that in vivo this stimulates beta cell compensation for insulin resistance, thus contributing to improved beta cell function. Nevertheless, data from freshly isolated islets clearly show an effect of PBA on insulin secretion.

Following 48 h high glucose exposure, increased insulin secretion was observed at basal (6.5 mmol/l) glucose in vivo, and in isolated islets, and this was not affected by TPO or PBA. This increase was not observed in islets at 2.8 mmol/l glucose, which suggests that it is likely not to be due to islet hypertrophy, which we have previously shown after 48 h glucose infusion [10], but to a shift in the sensitivity of the beta cell to glucose, possibly due to induction [41, 42] and/or increased activity of glycolytic genes as previously shown in a glucose-infusion model [42].

Previously, we reported that 48 h hyperglycaemia does not increase islet cell apoptosis or affect viability [10]. Thus, these findings suggest that our 48 h model is mainly a model of glucose-induced beta cell dysfunction rather than loss [10]. However, in the present study, with 48 h hyperglycaemia, an increase in *Chop* mRNA was observed. This effect was modest and both TPO and PBA prevented this increase. These findings warrant the investigation of glucose-induced beta cell loss in more chronic models of glucotoxicity. We have attempted to measure CHOP protein by western blotting and immunohistochemistry, but the results were

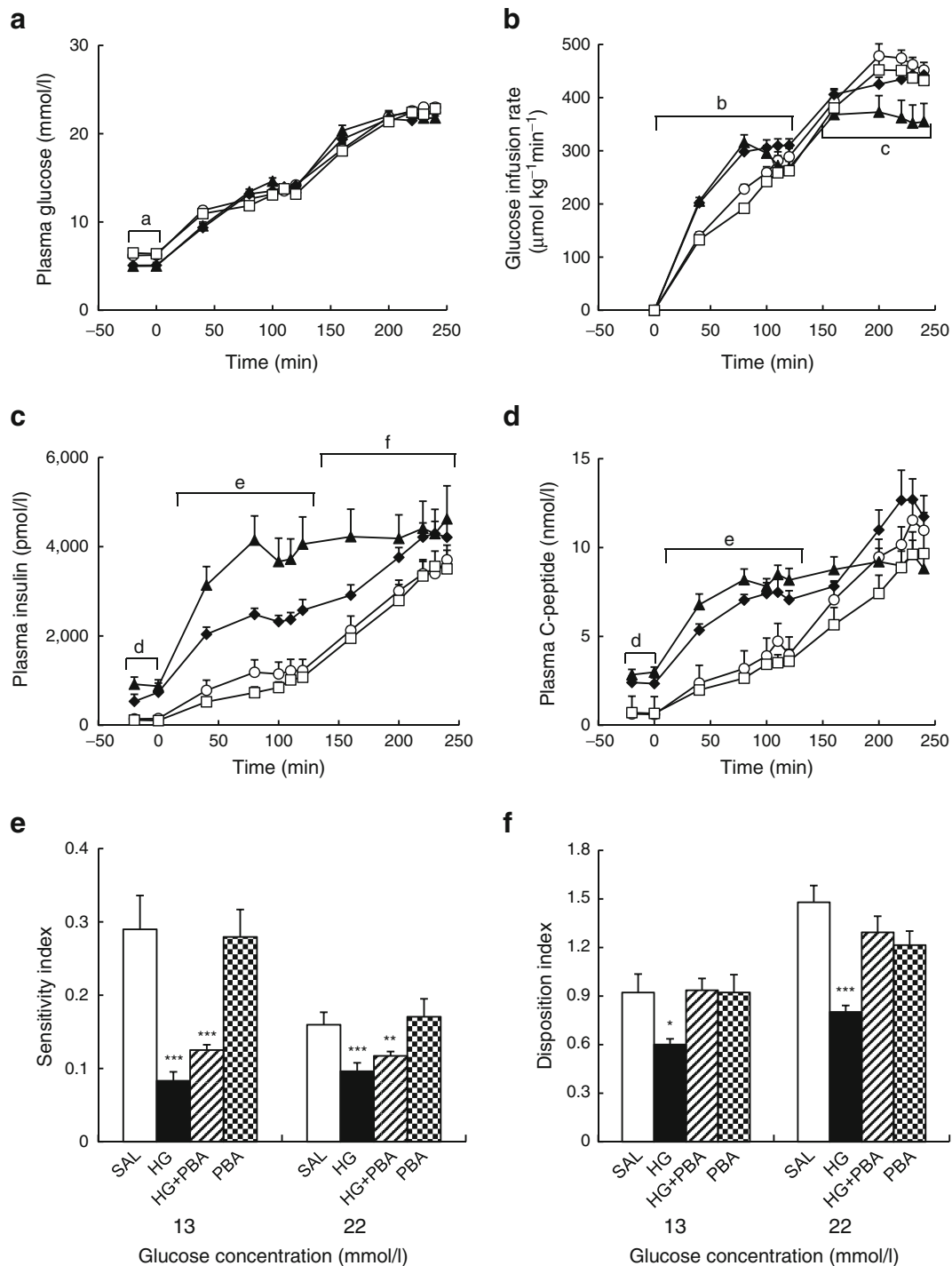


Fig. 8 PBA prevents glucose-induced beta cell dysfunction in vivo during a two-step hyperglycaemic clamp. The groups are described in the legend of Fig. 4. At time=0, an infusion of 37.5% glucose was started. Plasma glucose was maintained at 13 mmol/l by adjusting the rate of glucose infusion according to frequent (5–10 min) glycaemic determinations. At 120 min, the glucose infusion was further raised to 22 mmol/l until the end of the experiment (time=240 min). Samples for insulin and C-peptide were taken at regular intervals. **a** Plasma glucose levels; **b** Ginf; **c** plasma insulin; **d** plasma C-peptide; **e**

sensitivity index (M/I index = $G_{inf}/\text{insulin}$); and **f** disposition index ($C\text{-peptide} \times M/I$ index), during the two-step hyperglycaemic clamp (SAL, $n=12$; HG, $n=8$; HG + PBA, $n=10$; PBA, $n=8$). Data are means \pm SE. ^a $p<0.01$ HG vs SAL, $p<0.001$ HG + PBA vs SAL at basal; ^b $p<0.05$ HG + PBA vs SAL at 13 mmol/l; ^c $p<0.05$ HG vs SAL at 22 mmol/l; ^d $p<0.001$ HG, HG + PBA vs SAL at basal; ^e $p<0.001$ HG, HG + PBA vs SAL at 13 mmol/l; ^f $p<0.05$ HG vs SAL at 22 mmol/l. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ vs SAL. **a–d**: SAL, white circles; HG, black triangles; HG + PBA, black diamonds; PBA, white squares

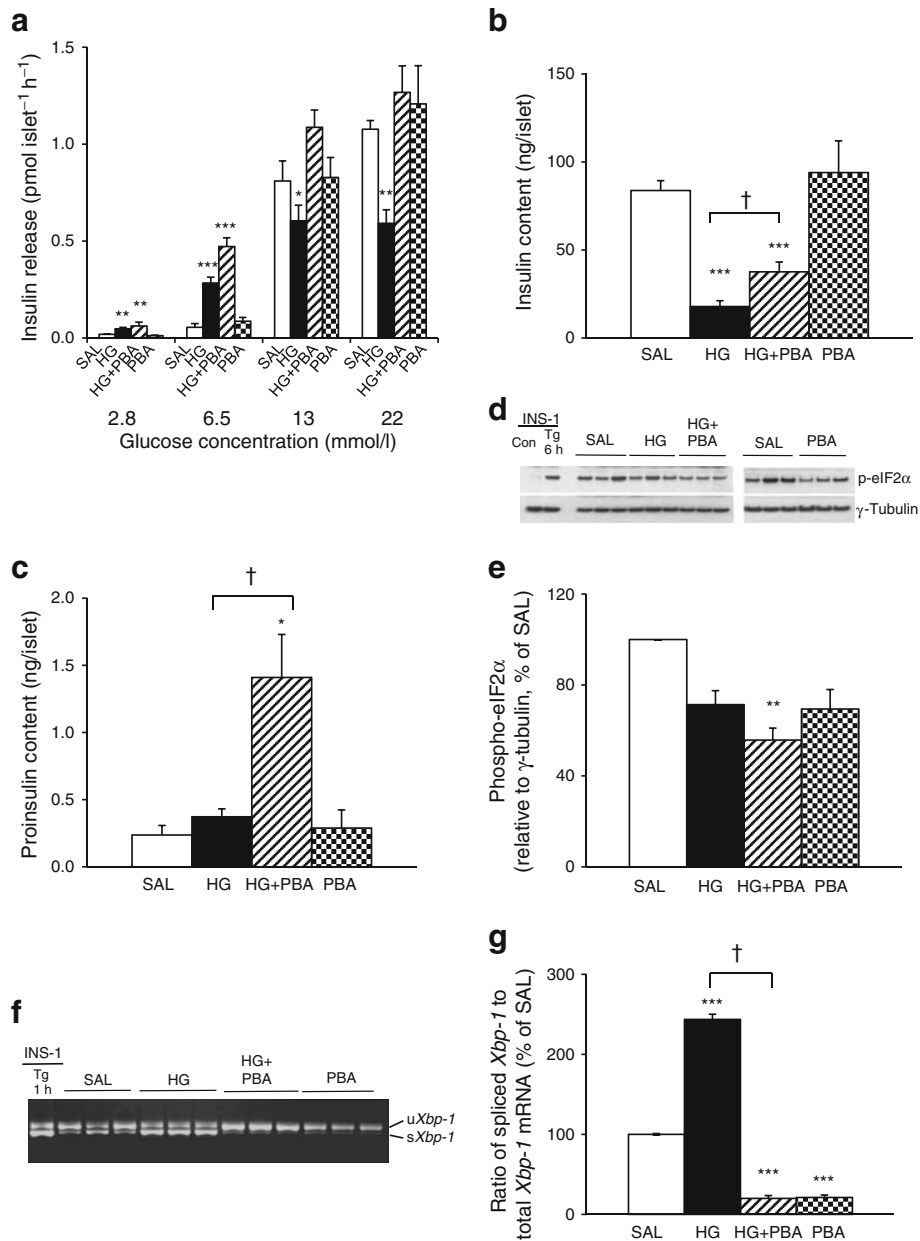


Fig. 9 PBA prevents glucose-induced beta cell dysfunction ex vivo following 96 h hyperglycaemia. Rats were treated for 96 h with: SAL; HG to maintain glycaemia at ~ 15 mmol/l; HG + PBA, $2.08 \mu\text{mol kg}^{-1} \text{min}^{-1}$; or PBA, $2.08 \mu\text{mol kg}^{-1} \text{min}^{-1}$. Following 96 h infusion, islets were isolated and pre-incubated in KRBH containing 2.8 mmol/l glucose for 1 h at 37°C . Thereafter, five islets of approximately the same size were transferred in triplicate to vials containing fresh medium plus glucose at the following concentrations: 2.8, 6.5, 13 and 22 mmol/l. Islets were incubated for 2 h at 37°C , and the insulin released was measured in the supernatant fraction (SAL, $n=8$; HG, $n=5$; HG + PBA, $n=6$; PBA, $n=4$) (a). The five islet pellets were subjected to acid-ethanol extraction for measurement of insulin content using RIA (SAL, $n=6$; HG, $n=5$; HG + PBA, $n=6$; PBA, $n=3$) (b) and proinsulin content using ELISA (SAL, $n=6$; HG, $n=5$; HG + PBA, $n=5$;

PBA, $n=3$) (c). **d** A representative immunoblot of phospho-eIF2 α (an indicator of PERK activation). Each lane represents islet lysates from one animal. As a positive control, cultured INS-1 cells were treated or not with thapsigargin for 6 h. Western blots for phospho-eIF2 α were quantified and results are expressed as % of SAL normalised by γ -tubulin (e) (SAL, $n=4$; HG, $n=3$; HG + PBA, $n=3$; PBA, $n=3$). **f** *Xbp1* mRNA was reverse transcribed and *uXbp1* and *sXbp1* were amplified by real-time PCR using primers that flank the intron excised by IRE-1 endoribonuclease activity. Each lane represents islet lysates from one animal. As a positive control, cultured INS-1 cells were treated with thapsigargin for 1 h. **g** The mean of the ratio of *sXbp1* to total *Xbp1* mRNA (as a % of SAL; SAL, $n=3$; HG, $n=5$; HG + PBA, $n=4$; PBA, $n=3$). Data are means \pm SE. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ vs SAL; † $p<0.05$ HG + PBA vs HG. Con, control; Tg, thapsigargin

variable due to the well-known specificity problem of the CHOP antibody [43]. In the absence of demonstrated CHOP

protein elevation or apoptosis, one may argue that glucose may not induce ER stress, but perhaps non-specific

upregulation of the UPR. However, we have recently been able to show increased levels of polyubiquitinated proteins in islets of HG-treated rats that are characteristic of misfolded and/or aggregated proteins (ESM Fig. 3). The role of hyperglycaemia in inducing ER stress can also be questioned because ER stress-induced CHOP production is not increased in the pancreas of type 1 diabetic individuals [44]. However, it should be considered that: (1) the role of ER stress in beta cell dysfunction and death in type 1 diabetes is minor in comparison with the role of cytokines, whereas their roles are reversed in the glucose infusion model; and (2) type 1 diabetic individuals are treated with insulin (beta cell rest), in contrast to beta cell overstimulation in glucose-infused rats. Regarding overstimulation in response to insulin resistance, Huang et al. have shown greater perinuclear CHOP in the pancreases of obese individuals [44], and greater nuclear CHOP in individuals with type 2 diabetes where ER stress may result from a combination of insulin overstimulation and hyperglycaemia.

In conclusion, our study suggests that ER stress plays a causal role in glucose-induced beta cell dysfunction *in vivo*, and that there is a reciprocal link between oxidative stress and ER stress in glucose-induced beta cell dysfunction *in vivo*. Furthermore, this study suggests that chemical chaperones are of potential interest to preserve beta cell function in type 2 diabetes.

Acknowledgements The authors thank L. Lam (Department of Physiology, University of Toronto) for her excellent technical assistance.

Funding This work was supported by Canadian Institutes of Health Research grants MOP-69018 (A. Giacca) and MOP-86641 (A. Volchuk). C. Tang was supported by Scholarships from the Banting and Best Diabetes Centre (University of Toronto), Ontario Graduate Scholarship, and the Canada Graduate Scholarship (Canadian Institutes of Health Research).

Contribution statement CT researched and analysed data and drafted the manuscript. KK researched and analysed data and contributed to revising and editing the manuscript. IS, LZ, TD, AI and PW researched and analysed data and contributed to revising the manuscript. CR-D, MBW and BM contributed to data analysis and revision of the manuscript. AV contributed to the design of the study and to the discussion, and reviewed and edited the manuscript. AG designed the study, contributed to the discussion, reviewed and finalised the manuscript. All the authors gave final approval to the submission of the manuscript.

Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

References

- Robertson RP (2004) Chronic oxidative stress as a central mechanism for glucose toxicity in pancreatic islet beta cells in diabetes. *J Biol Chem* 279:42351–42354
- Zhang K, Kaufman RJ (2008) From endoplasmic-reticulum stress to the inflammatory response. *Nature* 454:455–462
- Wang H, Kouri G, Wollheim CB (2005) ER stress and SREBP-1 activation are implicated in beta-cell glucolipotoxicity. *J Cell Sci* 118:3905–3915
- Elouil H, Bensellam M, Guiot Y et al (2007) Acute nutrient regulation of the unfolded protein response and integrated stress response in cultured rat pancreatic islets. *Diabetologia* 50:1442–1452
- Lipson KL, Fonseca SG, Ishigaki S et al (2006) Regulation of insulin biosynthesis in pancreatic beta cells by an endoplasmic reticulum-resident protein kinase IRE1. *Cell Metab* 4:245–254
- Hou ZQ, Li HL, Gao L, Pan L, Zhao JJ, Li GW (2008) Involvement of chronic stresses in rat islet and INS-1 cell glucotoxicity induced by intermittent high glucose. *Mol Cell Endocrinol* 291:71–78
- Qiu Y, Mao T, Zhang Y et al. A crucial role for RACK1 in the regulation of glucose-stimulated IRE1 α activation in pancreatic beta cells. *Sci Signal* 3 (106): ra7
- Cunha DA, Hekerman P, Ladriere L et al (2008) Initiation and execution of lipotoxic ER stress in pancreatic beta-cells. *J Cell Sci* 121:2308–2318
- Zhang L, Lai E, Teodoro T, Volchuk A (2009) GRP78, but not protein-disulfide isomerase, partially reverses hyperglycaemia-induced inhibition of insulin synthesis and secretion in pancreatic β cells. *J Biol Chem* 284:5289–5298
- Tang C, Han P, Oprea AI et al (2007) Evidence for a role of superoxide generation in glucose-induced beta-cell dysfunction *in vivo*. *Diabetes* 56:2722–2731
- Laybutt DR, Preston AM, Akerfeldt MC et al (2007) Endoplasmic reticulum stress contributes to beta cell apoptosis in type 2 diabetes. *Diabetologia* 50:752–763
- Del Guerra S, Lupi R, Marselli L et al (2005) Functional and molecular defects of pancreatic islets in human type 2 diabetes. *Diabetes* 54:727–735
- Back SH, Scheuner D, Han J et al (2009) Translation attenuation through eIF2 α phosphorylation prevents oxidative stress and maintains the differentiated state in beta cells. *Cell Metab* 10:13–26
- Song B, Scheuner D, Ron D, Pennathur S, Kaufman RJ (2008) Chop deletion reduces oxidative stress, improves beta cell function, and promotes cell survival in multiple mouse models of diabetes. *J Clin Invest* 118:3378–3389
- Carducci MA, Gilbert J, Bowling MK et al (2001) A Phase I clinical and pharmacological evaluation of sodium phenylbutyrate on a 120-h infusion schedule. *Clin Cancer Res* 7:3047–3055
- Welch WJ, Brown CR (1996) Influence of molecular and chemical chaperones on protein folding. *Cell Stress Chaperones* 1:109–115
- Hertl M, Hertl MC, Malago M, Broelsch CE (1999) *In vivo* protection of the pig liver against ischemia/reperfusion injury by tauroursodeoxycholate. *Langenbecks Arch Surg* 384:461–466
- Kaniuk NA, Kiraly M, Bates H, Vranic M, Volchuk A, Brumell JH (2007) Ubiquitinated-protein aggregates form in pancreatic beta-cells during diabetes-induced oxidative stress and are regulated by autophagy. *Diabetes* 56:930–939
- Asfari M, Janjic D, Meda P, Li G, Halban PA, Wollheim CB (1992) Establishment of 2-mercaptoethanol-dependent differentiated insulin-secreting cell lines. *Endocrinology* 130:167–178
- Lai E, Teodoro T, Volchuk A (2007) Endoplasmic reticulum stress: signaling the unfolded protein response. *Physiology (Bethesda)* 22:193–201
- Ron D, Walter P (2007) Signal integration in the endoplasmic reticulum unfolded protein response. *Nat Rev Mol Cell Biol* 8:519–529
- Malhotra JD, Miao H, Zhang K et al (2008) Antioxidants reduce endoplasmic reticulum stress and improve protein secretion. *Proc Natl Acad Sci USA* 105:18525–18530

23. Pizzo P, Pozzan T (2007) Mitochondria-endoplasmic reticulum choreography: structure and signaling dynamics. *Trends Cell Biol* 17:511–517
24. Scheuner D, Kaufman RJ (2008) The unfolded protein response: a pathway that links insulin demand with beta-cell failure and diabetes. *Endocr Rev* 29:317–333
25. Cardozo AK, Ortis F, Storling J et al (2005) Cytokines downregulate the sarcoendoplasmic reticulum pump Ca²⁺ ATPase 2b and deplete endoplasmic reticulum Ca²⁺, leading to induction of endoplasmic reticulum stress in pancreatic beta-cells. *Diabetes* 54:452–461
26. Hennige AM, Lember N, Wahl MA, Ammon HP (2000) Oxidative stress increases potassium efflux from pancreatic islets by depletion of intracellular calcium stores. *Free Radic Res* 33:507–516
27. Tu BP, Weissman JS (2002) The FAD- and O(2)-dependent reaction cycle of Ero1-mediated oxidative protein folding in the endoplasmic reticulum. *Mol Cell Biol* 22:983–994
28. Grolach A, Klappa P, Kietzmann T (2006) The endoplasmic reticulum: folding, calcium homeostasis, signaling, and redox control. *Antioxid Redox Signal* 8:1391–1418
29. Rutkowski DT, Arnold SM, Miller CN et al (2006) Adaptation to ER stress is mediated by differential stabilities of pro-survival and pro-apoptotic mRNAs and proteins. *PLoS Biol* 4:e374
30. Zhang P, McGrath B, Li S et al (2002) The PERK eukaryotic initiation factor 2 alpha kinase is required for the development of the skeletal system, postnatal growth, and the function and viability of the pancreas. *Mol Cell Biol* 22:3864–3874
31. Ma Y, Hendershot LM (2004) Herp is dually regulated by both the endoplasmic reticulum stress-specific branch of the unfolded protein response and a branch that is shared with other cellular stress pathways. *J Biol Chem* 279:13792–13799
32. Harding HP, Zhang Y, Zeng H et al (2003) An integrated stress response regulates amino acid metabolism and resistance to oxidative stress. *Mol Cell* 11:619–633
33. Boden G, Ruiz J, Kim CJ, Chen X (1996) Effects of prolonged glucose infusion on insulin secretion, clearance, and action in normal subjects. *Am J Physiol* 270:E251–E258
34. Giacca A, Xiao C, Oprescu AI, Carpentier AC, Lewis GF (2011) Lipid-induced pancreatic beta-cell dysfunction: focus on in vivo studies. *Am J Physiol Endocrinol Metab* 300:E255–E262
35. Urano F, Wang X, Bertolotti A et al (2000) Coupling of stress in the ER to activation of JNK protein kinases by transmembrane protein kinase IRE1. *Science* 287:664–666
36. Kaneto H, Xu G, Fujii N, Kim S, Bonner-Weir S, Weir GC (2002) Involvement of c-Jun N-terminal kinase in oxidative stress-mediated suppression of insulin gene expression. *J Biol Chem* 277:30010–30018
37. Andreozzi F, D'Alessandris C, Federici M et al (2004) Activation of the hexosamine pathway leads to phosphorylation of insulin receptor substrate-1 on Ser307 and Ser612 and impairs the phosphatidylinositol 3-kinase/Akt/mammalian target of rapamycin insulin biosynthetic pathway in RIN pancreatic beta-cells. *Endocrinology* 145:2845–2857
38. Miwa I, Ichimura N, Sugiura M, Hamada Y, Taniguchi S (2000) Inhibition of glucose-induced insulin secretion by 4-hydroxy-2-nonenal and other lipid peroxidation products. *Endocrinology* 141:2767–2772
39. Krauss S, Zhang CY, Scorrano L et al (2003) Superoxide-mediated activation of uncoupling protein 2 causes pancreatic beta cell dysfunction. *J Clin Invest* 112:1831–1842
40. Ozcan U, Yilmaz E, Ozcan L et al (2006) Chemical chaperones reduce ER stress and restore glucose homeostasis in a mouse model of type 2 diabetes. *Science* 313:1137–1140
41. Roche E, Assimacopoulos-Jeannet F, Witters LA et al (1997) Induction by glucose of genes coding for glycolytic enzymes in a pancreatic beta-cell line (INS-1). *J Biol Chem* 272:3091–3098
42. Chen C, Bumbalo L, Leahy JL (1994) Increased catalytic activity of glucokinase in isolated islets from hyperinsulinemic rats. *Diabetes* 43:684–689
43. Haataja L, Gurlo T, Huang CJ, Butler PC (2008) Many commercially available antibodies for detection of CHOP expression as a marker of endoplasmic reticulum stress fail specificity evaluation. *Cell Biochem Biophys* 51:105–107
44. Huang CJ, Lin CY, Haataja L et al (2007) High expression rates of human islet amyloid polypeptide induce endoplasmic reticulum stress mediated beta-cell apoptosis, a characteristic of humans with type 2 but not type 1 diabetes. *Diabetes* 56:2016–2027