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ARTICLE

Endoplasmic reticulum stress contributes to beta cell apoptosis in type 2 diabetes

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Abstract
Aims/hypothesis Increased lipid supply causes beta cell death, which may contribute to reduced beta cell mass in type 2 diabetes. We investigated whether endoplasmic reticulum (ER) stress is necessary for lipid-induced apoptosis in beta cells and also whether ER stress is present in islets of an animal model of diabetes and of humans with type 2 diabetes.
Methods Expression of genes involved in ER stress was evaluated in insulin-secreting MIN6 cells exposed to elevated lipids, in islets isolated from *db*/*db* mice and in pancreas sections of humans with type 2 diabetes. Overproduction of the ER chaperone heat shock 70 kDa protein 5 (HSPA5, previously known as immunoglobulin heavy chain binding protein [BIP]) was performed to assess whether attenuation of ER stress affected lipid-induced apoptosis.
Results We demonstrated that the pro-apoptotic fatty acid palmitate triggers a comprehensive ER stress response in MIN6 cells, which was virtually absent using non-apoptotic fatty acid oleate. Time-dependent increases in mRNA levels for activating transcription factor 4 (*Atf4*), DNA-damage inducible transcript 3 (*Ddit3*, previously known as C/EBP homologous protein [*Chop*]) and DnaJ homologue (HSP40) C3 (*Dnajc3*, previously known as *p58*) correlated with increased apoptosis in palmitate- but not in oleate-treated MIN6 cells. Attenuation of ER stress by overproduction of HSPA5 in MIN6 cells significantly protected against lipid-induced apoptosis. In islets of *db*/*db* mice, a variety of marker genes of ER stress were also upregulated. Increased processing (activation) of X-box binding protein 1 (*Xbp1*) mRNA was also observed, confirming the existence of ER stress. Finally, we observed increased islet protein production of HSPA5, DDIT3, DNAJC3 and BCL2-associated X protein in human pancreas sections of type 2 diabetes subjects.
Conclusions/interpretation Our results provide evidence that ER stress occurs in type 2 diabetes and is required for aspects of the underlying beta cell failure.

Keywords Apoptosis · Endoplasmic reticulum stress · Fatty acids · Islets · Pancreatic beta cells · Type 2 diabetes

Abbreviations

ATF	activating transcription factor
BAX	BCL2-associated X protein
BCL2	B-cell CLL/lymphoma 2
CREBL1	cAMP responsive element binding protein-like 1
DDIT3	DNA-damage inducible transcript 3
DNAJC3	DnaJ (Hsp40) homologue C3

D. R. Laybutt and A. M. Preston have contributed equally to this work.

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EIF2A	eukaryotic translation initiation factor 2A
EIF2AK3	eukaryotic translation initiation factor kinase 2-alpha kinase 3
ER	endoplasmic reticulum
GFP	green fluorescent protein
HSPA5	heat shock 70 kDa protein 5
IRE1	inositol requiring enzyme 1
MAPK8	mitogen-activated protein kinase 8
PDIA4	protein disulfide isomerase A4
UPR	unfolded protein response
XBP1	X-box binding protein 1

Introduction

Type 2 diabetes results from the failure of pancreatic beta cells to adequately compensate for obesity and insulin resistance. Both functional defects and reduced beta cell mass contribute to beta cell failure in type 2 diabetes, with apoptosis constituting the main form of beta cell death [1–4]. Increased lipids and hyperglycaemia are likely causes of beta cell apoptosis [3–6], but the mechanisms responsible remain unknown.

Pancreatic beta cells possess a highly developed endoplasmic reticulum (ER), required for the folding, export and processing of newly synthesised insulin [7, 8]. Various conditions that disrupt ER function, termed ER stress, lead to the accumulation of misfolded proteins in the ER [7–9]. This triggers an adaptive programme comprising four distinct responses: (1) translational attenuation, which reduces synthesis of new protein and prevents further accumulation of unfolded proteins; (2) upregulation of the genes encoding ER chaperone proteins to increase protein folding activity and to prevent protein aggregation; (3) proteosomal degradation of misfolded proteins following their regulated extrusion from the ER; and (4) apoptosis in the event of persistent stress. The signalling pathways that underlie this programme and relay information from the ER to the nucleus are known as the unfolded protein response (UPR). Heat shock 70 kDa protein 5 (HSPA5, previously known as immunoglobulin heavy chain binding protein [BIP]) is central to this process as it serves both as an ER chaperone and a sensor of protein misfolding [10]. In non-stressed cells, HSPA5 associates on the ER luminal surface with three UPR transducer proteins, inositol requiring enzyme 1 (IRE1), activating transcription factor (ATF) 6 and eukaryotic translation initiation factor kinase 2-alpha kinase 3 (EIF2AK3, formerly known as PKR-like ER kinase [PERK]), thus maintaining them in inactive conformations. Under stressed conditions, HSPA5 dissociates from the transducer proteins, inducing their activation and subsequent upregulation of UPR target genes, as well as translational attenuation due to phosphorylation of the eukaryotic translation initiation factor 2A (EIF2A) by the protein kinase EIF2AK3. EIF2A, however, is also a substrate for other protein kinases activated during the so-called integrated stress response. When ER function is severely impaired, apoptosis is induced by enhanced transcription of DNA-damage inducible transcript 3 (DDIT3, previously known as C/EBP homologous protein [CHOP]) [11, 12] and by activation of mitogen-activated protein kinase 8 (MAPK8, formerly known as JNK1) and caspase-12 [7–9].

Using *Eif2ak3*-deficient mice [13], and in mice with a mutation in the EIF2A phosphorylation site (Ser51Ala) [14, 15], it has been demonstrated that beta cells are particularly sensitive to ER stress-induced dysfunction and death. Furthermore, studies in the Akita mouse have shown that ER stress, secondary to misfolding of mutated insulin, leads to beta cell death and glucose intolerance [12]. Recent studies have also shown that pre-treatment of INS-1 beta cells with fatty acid leads to increased expression of several genes involved in ER stress [16, 17]. By comprehensive profiling of gene expression, we now demonstrate that saturated fatty acid induces an extensive ER stress response in MIN6 cells and that this is required for the accompanying apoptosis. Furthermore, we provide the first evidence of significant ER stress gene activation in pancreatic islets of diabetic *db*/*db* mice and humans with type 2 diabetes.

Materials and methods

Cell culture and treatment MIN6 cells were passaged in 150 cm^2 flasks with 25 ml DMEM (Invitrogen, Carlsbad, CA, USA) containing 25 mmol/l glucose, 10 mmol/l HEPES, 10% FCS, 50 U/ml penicillin and 50 μg/ml streptomycin. Cells were seeded at either 1×10^6 in 3 ml of DMEM per well in a six-well plate or at 2.6×10^5 in 0.5 ml DMEM per well in a 24-well plate. After 24 h, the medium was replaced with DMEM as above but with 6 mmol/l glucose containing either BSA or BSA coupled to oleate or palmitate (1:20 coupling; DMEM, final concentration 0.4 mmol/l fatty acid; 0.92% BSA) as previously described [18]. Apoptosis was measured with an ELISA kit (Cell Death Detection ELISA; Roche Diagnostics, Castle Hill, NSW, Australia) [19].

RNA analysis Total RNA was extracted from MIN6 cells or islets [18] and real-time PCR was performed using a LightCycler (Roche Diagnostics) [20]. Standards for each transcript were prepared in a conventional PCR and purified using a PCR product purification kit (High Pure; Roche Diagnostics). The value obtained for each specific product was normalised to a control gene (cyclophilin A) and expressed as a percentage of the value in control

extracts. Transcript profiling data that had been previously reported [18] were re-analysed here using MAS 5.0 software (Affymetrix, Santa Clara, CA, USA).

Generation of HSPA5-overproducing MIN6 cells Murine *Hspa5* cDNA was amplified by PCR, cloned into the Gateway donor vector pDONR 221 and then subcloned into the Gateway expression vector pcDNA-DEST40 (Invitrogen). Phospho-*Hspa5*-DEST40 or control pmaxGFP were electroporated into MIN6 cells by nucleofection (AMAXA Biosystems, Cologne, Germany) according to the manufacturers' instructions with >70% transfection efficiency. Cells were seeded at 5×10^5 in 1 ml of DMEM per well in a 12-well plate. After 24 h, the medium was replaced with DMEM with 6 mmol/l glucose and either BSA or BSA coupled to palmitate for 48 h.

Animals C57BL/KsJ *db*/*db* mice and their age-matched lean *db*/+ littermates (control) were bred inhouse using animals originally from Jackson Laboratories (Bar Harbor, ME, USA). Procedures were approved by the Garvan Institute/St Vincent's Hospital Animal Experimentation Ethics Committee, following guidelines issued by the National Health and Medical Research Council of Australia. Non-diabetic *db*/+ and diabetic *db*/*db* mice aged 10 to 12 weeks were anaesthetised and their islets isolated by pancreatic digestion with liberase RI (Roche Diagnostics). Islets were further separated with a Ficoll–Paque PLUS gradient (GE Healthcare Bio-Sciences, Uppsala, Sweden) and handpicked under a stereomicroscope. RNA or protein extraction was performed immediately following islet collection.

Western blotting Cell and islet extracts were separated on NuPage SDS-PAGE gels (Invitrogen) and transferred to polyvinylidine difluoride membranes. Equal loading of protein between lanes was confirmed by Coomassie staining and subsequent β-actin immunoblots. Membranes were incubated in primary antibodies diluted in 5% BSA in Tris-buffered saline with 0.05% Tween for either 1 to 2 h at room temperature or overnight at 4°C. The following antibodies were used (1:1,000 dilution unless otherwise indicated): DDIT3 (sc-575), total EIF2A (sc-11386), and X-box binding protein 1 (XBP1; sc-7160; Santa Cruz Biotechnology, Santa Cruz, CA, USA); phospho-EIF2AK3 (Thr980, 3191), phospho-EIF2A (Ser51, 9721), cleaved caspase-3 (Asp175, 9664, 1:500; Cell Signaling Technology, Danvers, MA, USA); HSPA5 (SPA-826; Stressgen, Victoria, BC, Canada); ATF6 (1:330; Alexis Biochemicals, San Diego, CA, USA); β-actin (1:5,000; Sigma, St Louis, MO, USA); and myosin (Biomedical Technologies, Stoughton, MA, USA). DNAJC3 (1:4,000) was generously provided by A. Goodman, University of Washington, WA, USA. After incubation with horseradish peroxidase-conjugated goat anti-mouse or donkey anti-rabbit antibody (1:5,000; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 1 h at room temperature, immunodetection was performed by chemiluminescence (PerkinElmer, Wellesley, MA, USA).

Tissue microarrays Archival formalin-fixed, paraffin-embedded tissue was collected from Westmead Hospital, St Vincent's Hospital Campus, Concord Hospital and Royal Prince Alfred Hospital in Sydney, Australia. The tissue sources were 11 type 2 diabetes patients and 12 non-diabetic patients, as classified from medical records, and whose pancreases were resected between 1990 and 2002. Ethical clearance was obtained from the participating Institutional Ethics Committees. Pancreas tissue microarrays consisting of 2-mm diameter tissue core biopsies containing islets were constructed. Serial sections (4 μm) were dewaxed in xylene and rehydrated in a series of graded alcohols. To unmask antigens slides were boiled in Tris–EDTA (pH 8) for 25 min. Slides were stained for HSPA5, DDIT3 and DNAJC3 using the antibodies described above, and antibodies for BCL2-associated X protein (BAX; 554104, dilution 1:1,000; BD PharMingen, San Diego, CA, USA), B cell CLL/lymphoma 2 (BCL2; M0887, dilution 1:50; DakoCytomation, Glostrup, Denmark) and insulin (I2018; dilution 1:200; Sigma). The primary antibody was visualised using EnVision+System-HRP DAB (Dako). Staining was independently assessed by two observers (D.R. Laybutt and M.C. Åkerfeldt); islet immunostaining was graded as of low, moderate or high intensity.

Statistical analysis All results are presented as means± SEM. Statistical analyses were performed using unpaired Student's *t* test or one-way ANOVA.

Results

Time-course changes in apoptosis in MIN6 cells exposed to elevated lipids As with primary beta cells, chronic exposure of the MIN6 cell line [21] to elevated fatty acids leads to secretory defects and enhanced apoptosis, which are reminiscent of the beta cell phenotype displayed in type 2 diabetes [3–6, 22]. Using MIN6 cells cultured with the saturated fatty acid palmitate, apoptosis was unchanged at 4 h, tended to a slight increase at 24 h and was elevated by fourfold after 48 h (Fig. 1). In contrast, apoptosis was unaffected by exposure to the unsaturated fatty acid oleate (Fig. 1). This distinction between the effects of saturated

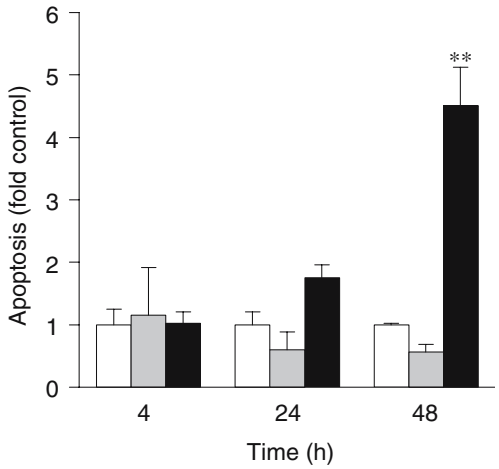

Fig. 1 Time-course changes in apoptosis in MIN6 cells exposed to palmitate or oleate. MIN6 cells were treated for 4, 24 or 48 h with either 0.92% BSA alone (*open bars*) or 0.92% BSA coupled to 0.4 mmol/l oleate (*grey bars*) or to 0.4 mmol/l palmitate (*dark bars*) and apoptosis was measured using a cell death detection ELISA kit. Results are means±SEM determined from three experiments performed in triplicate and are expressed as fold-change compared with control. $^{**}p<0.01$ vs control-treated MIN6 cells at the same time point

and unsaturated fatty acids has been previously identified [6, 23–26], although the underlying explanation is unclear.

Changes, in MIN6 cells exposed to elevated lipids, of mRNA levels of genes involved in ER stress The regulated expression of genes involved in ER stress by fatty acids in MIN6 cells was examined by real-time RT-PCR (oligonucleotide primers, see electronic supplementary Table 1). Exposure to palmitate induced time-dependent increases in *Atf4*, *Ddit3* and *Dnajc3* (Fig. 2a–c). Levels of *Atf4* mRNA, known to be induced downstream of EIF2A phosphorylation [27], were significantly induced as early as 4 h and were further augmented at 48 h, showing a significant time-dependency ($p<0.05$). *Ddit3* mRNA levels were also increased in a step-wise fashion with time ($p<0.002$). mRNA levels of *Dnajc3*, another ER stress protein induced predominately downstream of ATF6 and XBP1 [28, 29], were unchanged at 4 h, but increased at 24 and 48 h, displaying a significant time-dependent effect ($p<0.001$). mRNA levels of the ER chaperone *Hspa5*, and the protein disulphide isomerase family A member 4 (*Pdia4*, previously known as *Erp72*) were induced to a lesser extent and only after 48 h of palmitate treatment (Fig. 2d,e). Thus, the upregulation of genes involved in ER stress was associated with the duration of palmitate exposure. In contrast, these genes were unchanged or even reduced in MIN6 cells treated with oleate (Fig. 2a–e). Thus *Atf4, Ddit3*, *Dnajc3*, *Hspa5* and *Pdia4* mRNA levels were unaltered at all time points (Fig. 2a–e), except for decreases in *Dnajc3* at 4 h (Fig. 2c) and in *Hspa5* at 48 h (Fig. 2d). These results demonstrate that ER stress is activated in a time-dependent and selective manner by saturated, but not by unsaturated fatty acids in MIN6 beta cells.

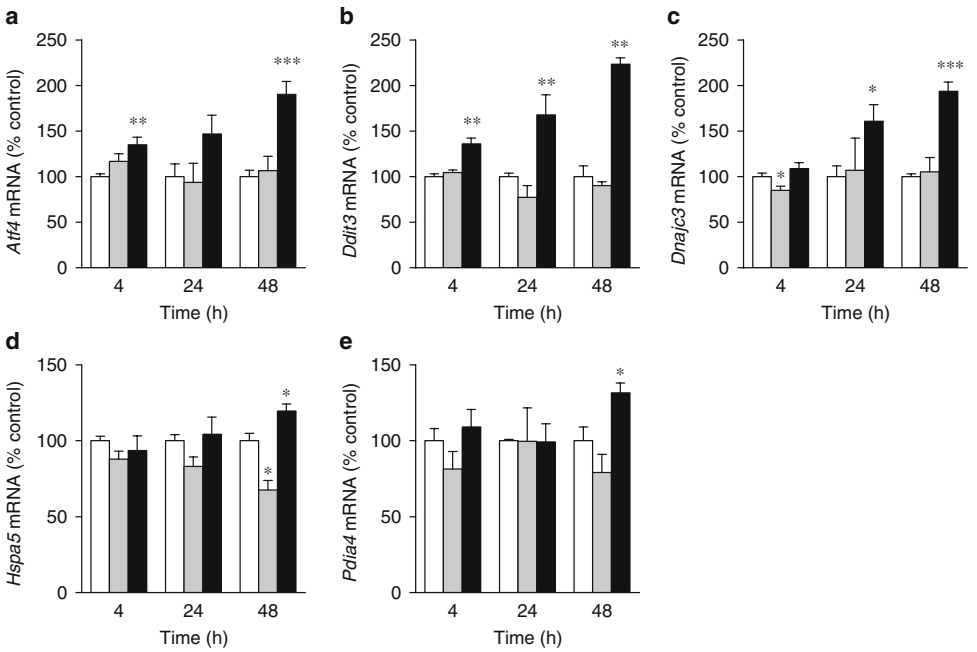

Fig. 2 Time-dependent upregulation of mRNA levels of genes involved in ER stress in palmitate-treated MIN6 cells. **a**–**e** MIN6 cells grown in six-well plates in DMEM were treated for 4, 24 or 48 h with either 0.92% BSA alone (*open bars*) or 0.92% BSA coupled to 0.4 mmol/l oleate (*grey bars*) or to 0.4 mmol/l palmitate (*dark bars*). Total RNA was extracted and analysed by real-time RT-PCR. Results are means±SEM determined from four to six experiments and are expressed as a percentage of mRNA levels in control-treated MIN6 cells. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ vs control-treated MIN6 cells at the same time point

Since our earlier study using gene-chips [18], many transcripts and expressed sequence tags have been re-annotated. Re-analysis of the data sets now revealed that palmitate induces a widespread ER stress response in MIN6 cells: 48 out of 235 transcripts increased by palmitate (≥1.5-fold, $p<0.05$) could be ascribed to this response (data not shown). These include UPR genes defined from studies in other mammalian cells [28–30] or genes known to mediate aspects of ER stress. Only six of these transcripts were also increased in oleate-treated cells, and always to a lesser extent than with palmitate (not shown). Changes in mRNA levels of representative genes were confirmed by RT-PCR (Table 1). This strongly reinforces the idea that the saturated fatty acid, palmitate, induces a comprehensive ER stress response that is associated with apoptosis, whereas neither feature is generated by the unsaturated fatty acid, oleate. Consistent with this conclusion, none of these ER stress transcripts were increased in palmitate-resistant cells (data not shown) that we had previously selected by chronic passaging in palmitate-containing media [19].

Time-course changes in ER stress markers in MIN6 cells exposed to elevated lipids To confirm that our findings were not merely due to the integrated stress response, we also examined EIF2AK3 phosphorylation, XBP1 splicing (54-kDa protein) and ATF6 cleavage, all of which are entirely dependent on activation of ER stress. In palmitate-treated MIN6 cells, both EIF2AK3 phosphorylation (Fig. 3a,b) and the active (54 kDa) form of the XBP1 protein (Fig. 3a,c), were increased at each time-point tested. In addition, generation of cleaved ATF6 (50 kDa protein) was increased transiently after 24 h of palmitate treatment (Fig. 3a,d). In contrast, these ER stress markers were unchanged or even reduced following treatment with oleate (Fig. 3a–d).

In further confirmation of the mRNA data, we observed that DDIT3 protein production was increased in a time-dependent manner by palmitate, but not by oleate (Fig. 3a,f). A similar fatty acid specificity was demonstrated for EIF2A (Ser51) phosphorylation in lipid-treated MIN6 cells, as assessed using phospho-specific antibodies (Fig. 3a,e). Thus the upregulation of UPR genes in response to palmitate precedes the accompanying beta cell apoptosis (Fig. 1).

HSPA5 overproduction partially protects MIN6 cells from lipid-induced apoptosis Overproduction of the ER chaperone HSPA5 attenuates ER stress, both by enhancing protein folding, and by helping to maintain IRE1, ATF6 and EIF2AK3 in their inactive states [10]. MIN6 cells were therefore transfected with an expression vector encoding for HSPA5 or green fluorescent protein (GFP; Fig. 4a) prior to lipid exposure for 48 h. Palmitate-induced apoptosis was significantly reduced in HSPA5-overproducing versus control (GFP-producing) MIN6 cells (Fig. 4b). HSPA5 overproduction also reduced ER stress due to palmitate, as indicated by reduced EIF2AK3 and EIF2A phosphorylation, and reduced production of active XBP1 (54 kDa protein), DDIT3 and cleaved caspase-3 (Fig. 4c). These data are the first indication that ER stress is at least partially required for lipid-induced apoptosis in beta cells.

Changes in mRNA levels for gene stress markers of ER in islets of db/db mice To confirm these results in an animal model of diabetes, we next examined the expression of genes encoding UPR in islets of *db*/*db* mice. These animals develop a disease resembling the common form of human type 2 diabetes whereby insulin secretory defects and beta cell depletion prevent compensation for time-dependent increases in obesity and insulin resistance [20, 31]. We found that *db*/*db* mice displayed increased body weight

Table 1 Changes to mRNA levels of genes involved in ER stress in MIN6 cells treated for 48 h with palmitate or oleate

Category	Gene description	Control	Palmitate	Oleate
Chaperone	*Hsp90b1* (*Grp94*)	100±6	159±16*	89±5
	Hyou1 (*Orp150*)	100±6	136±11*	79±1
Co-chaperone	*Dnajb9*	100±4	179±7***	90±3
Disulfide isomerase	*Qscn6* (*Qsox1*)	100±3	191±18***	105±1
Protein folding	*Fkbp11*	100±2	200±33*	85±6*
	Herpud1 (*Herp*)	100±7	163±12**	90±7
ERAD	*H47* (*Sels*, *Vimp*)	100±3	188±14***	76±4**
	Edem1	100±5	128±5**	80±4*

Data are means±SEM determined from four to six experiments and are expressed as a percentage of mRNA levels in control-treated MIN6 cells. *$p<0.05$, ** $p<0.01$, *** $p<0.001$ versus control-treated MIN6 cells

ERAD ER-associated degradation; *Hsp90b1*, heat shock protein 90 kDa beta, member 1, (previously known as *Grp94*); *Hyou1*, hypoxia upregulated 1 (previously known as *Orp150*); *Qscn6*, quiescin Q6; *Fkbp11*, FK506 binding protein 11; *Herpud1*, homocysteine-inducible, endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1 (previously known as *Herp*); *H47*, histocompatibility 47 (previously known as *Sels*, *Vimp*); *Edem1*, ER degradation enhancer, mannosidase alpha-like 1

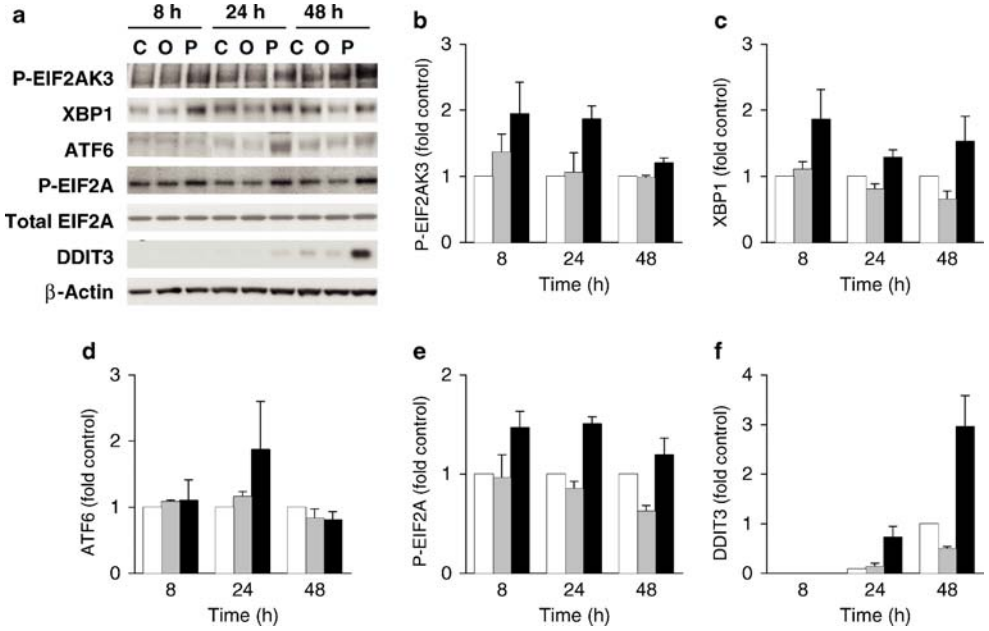

Fig. 3 Upregulated expression of genes involved in ER stress in palmitate-treated MIN6 cells. **a** representative western blot comparing time-course changes in EIF2AK3 and EIF2A phosphorylation (*P*), and production of activated XBP1 (54 kDa), cleaved (activated) ATF6 (50 kDa) and DDIT3 in MIN6 cells treated for 8, 24 or 48 h with either 0.92% BSA as control (*C*) or 0.92% BSA coupled to 0.4 mmol/l oleate (*O*) or to 0.4 mmol/l palmitate (*P*). Total EIF2A and β-actin protein served as loading controls. **b** P-EIF2AK3, **c** 54 kDa XBP, **d** 50 kDa ATF6, **e** P-EIF2A and **f** DDIT3 bands were quantitated by densitometry and are expressed as fold-change compared with control. Values are means±SEM determined from three to four experiments in control (*open bars*), oleate (*grey bars*) or palmitate (*dark bars*) treated MIN6 cells. Note, DDIT3 data were compared with values in the control at 48 h, since in some experiments no band was present in control-treated cells at earlier time points

(obesity), hyperglycaemia and higher circulating levels of lipid (NEFA and triacylglycerol) than lean *db*/+ control mice (not shown).

Using real-time RT-PCR, we determined that the expression of multiple UPR genes was enhanced in islets of 10- to 12-week-old *db*/*db* (diabetic) versus *db*/+ (control)

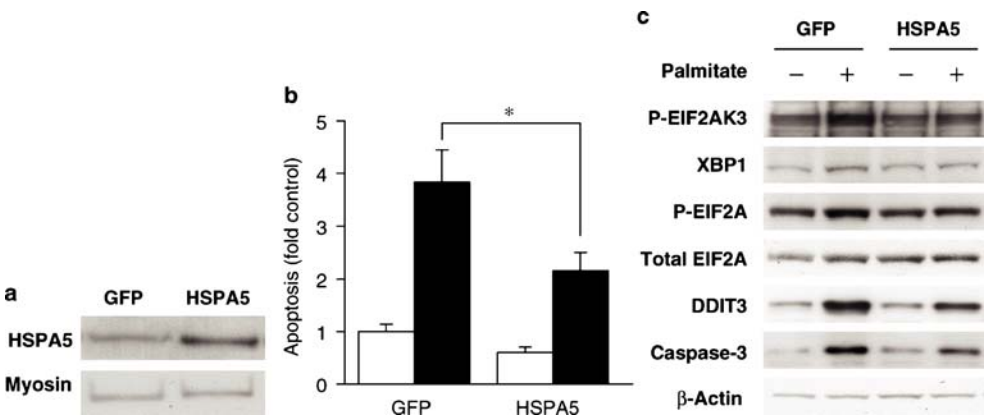

Fig. 4 HSPA5 overproduction partially protects MIN6 cells from lipid-induced apoptosis. **a** Western blot analysis of HSPA5 in GFP- and HSPA5-overproducing MIN6 cells at the end of the 48 h treatment period (72 h after transfection). MIN6 cells were transfected with an expression vector encoding for *Hspa5* (pBIP-DEST40) or GFP (pmaxGFP) by nucleofection. Myosin served as a loading control. **b** Effect of HSPA5 overproduction on lipid-induced apoptosis in MIN6 cells. 24 h after transfection, HSPA5- and GFP-overproducing MIN6 cells were treated with either 0.92% BSA alone (*open bars*) or 0.92% BSA coupled to 0.4 mmol/l palmitate (*dark bars*) for 48 h and apoptosis measured using a cell death detection ELISA kit. Results are means±SEM determined from three experiments performed in triplicate. *$p<0.05$ for difference between palmitate-treated GFP- vs palmitate-treated HSPA5-overproducing MIN6 cells. **c** Reduced activation of genes involved in ER stress in palmitate-treated HSPA5-overproducing MIN6 cells compared with palmitate-treated control (GFP) cells. Western blot analysis of phospho(P)-EIF2AK3, XBP1 (active 54 kDa form), P-EIF2A, total EIF2A, DDIT3, cleaved caspase-3 and β-actin in GFP- and HSPA5-overproducing MIN6 cells treated with either 0.92% BSA alone or 0.92% BSA coupled to 0.4 mmol/l palmitate for 48 h. Representative western blot images are shown from two to three experiments

mice (Fig. 5a,b). Indeed, these changes were generally larger than the corresponding increases observed in palmitate-treated MIN6 cells (Fig. 2, Table 1). An exception was *Ddit3* which showed only modest, though significant, induction in islets of *db*/*db* mice (Fig. 5a). mRNA levels for *Atf6* and cAMP responsive element binding protein-like 1 (*Crebl1* [formerly known as *Atf6β*]) were unchanged (Fig. 5a) as these transducers are predominately regulated by post-translational cleavage during ER stress. In confirmation of the mRNA data, western blotting revealed a sixfold increase in DNAJC3 protein production in islet extracts of diabetic mice compared with control (Fig. 6a,b). Moreover, phospho-specific antibodies demonstrated increased EIF2A (Ser51) phosphorylation in *db*/*db* islets (Fig. 6a,c). In order to differentiate these responses from the integrated stress response, we also took advantage of the fact that ER stress leads to splicing of *Xbp1* mRNA, resulting in a frame-shift, through which there is a rearrangement to an active form and the loss of a Pst1 restriction site [32]. We therefore examined *Xbp1* activation in *db*/*db* islets by PCR amplifying *Xbp1* cDNA followed by incubation with PST1. A significantly reduced proportion of *Xbp1* cDNA was shown to be cut by PST1 in islets of diabetic mice compared with control mice, and was thus indicative of *Xbp1* splicing and ER stress (Fig. 7a). In addition, using western blot, we observed an induction of the active form of XBP1 protein (54 kDa) in islets of *db*/*db* mice (Fig. 7b). These data provide the first demonstration of UPR activation and the presence of ER stress in islets of an animal model of type 2 diabetes.

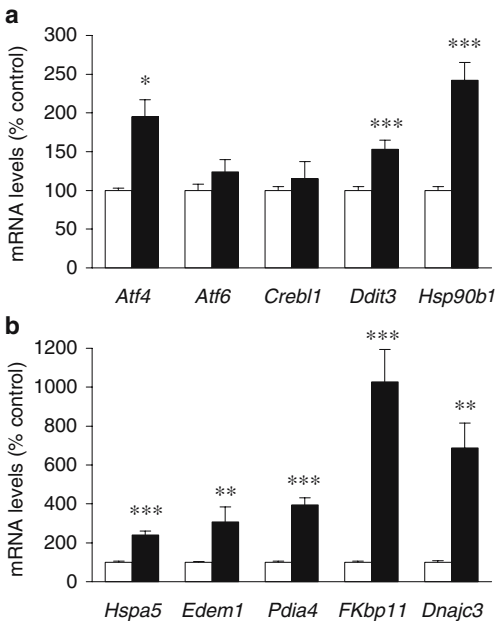

Fig. 5 Upregulated mRNA levels of genes involved in ER stress in islets of diabetic mice. **a***Atf4*, *Atf6*, *Crebl1*, *Ddit3* and *Hsp90b1*mRNA levels. **b***Hspa5*, ER degradation enhancer, mannosidase alpha-like 1 (*Edem1*), *Pdia4*, FK506 binding protein 11 (*Fkbp11*) and *Dnajc3* mRNA levels. Total RNA was extracted from islets isolated from control (*db*/+) and diabetic (*db*/*db*) mice and analysed by real-time RT-PCR. Results are expressed as a percentage of mRNA levels in control islets. Values are means±SEM determined from control (n=5–14; *open bars*) and *db*/*db* mice (n=5–10; *dark bars*). *p<0.05, **p<0.01, ***p<0.001 vs control for each gene

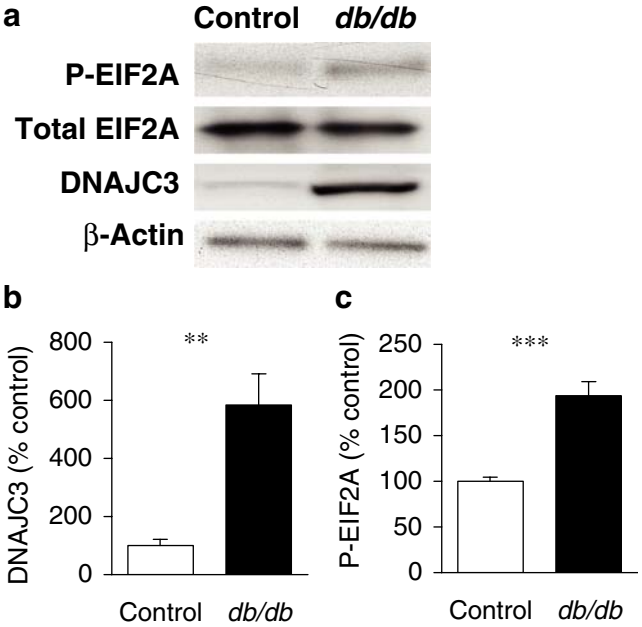

Fig. 6 Increased DNAJC3 production and EIF2A phosphorylation in islets of diabetic *db*/*db* mice. Islets isolated from lean control and diabetic *db*/*db* mice were lysed and subjected to SDS-PAGE and levels of phospho(P)-EIF2A and DNAJC3 assessed by immunoblotting. **a** Representative immunoblots of P-EIF2A and DNAJC3. Total EIF2A and β-actin are shown as a control of protein loading. **b** DNAJC3 and **c** P-EIF2A bands were quantitated by densitometry and are expressed as a percentage of control values. Values are means±SEM determined from five mice per group. **p<0.01; ***p<0.001

Increased DDIT3, HSPA5 and DNAJC3 staining in islets from human type 2 diabetes subjects Tissue microarrays were constructed from pancreas of human type 2 diabetes subjects and non-diabetic control subjects. These were sectioned and immunostained using antibodies for insulin, BAX, HSPA5, DDIT3 and DNAJC3. All islet immunostaining results were quantified according to immunointensity; each subject was graded as low, moderate or high and expressed as a percentage of total non-diabetic and diabetes subjects, respectively (Fig. 8). An immunoscore was calculated by assigning a scale of 1 (low), 2 (moderate) or 3 (high) to staining intensity. As expected, type 2 diabetes was accompanied by reduced immunostaining for insulin and enhanced production of the pro-apoptotic protein BAX (Fig. 8, Table 2), which is consistent with chronic beta cell exposure to elevated glucose and fatty acids [33]. The mean intensity of islet immunostaining was significantly higher in the type 2 diabetes subjects than in non-diabetic subjects for HSPA5, DDIT3, DNAJC3 and BAX proteins (Table 2). BCL2 staining was barely detectable in islets from non-

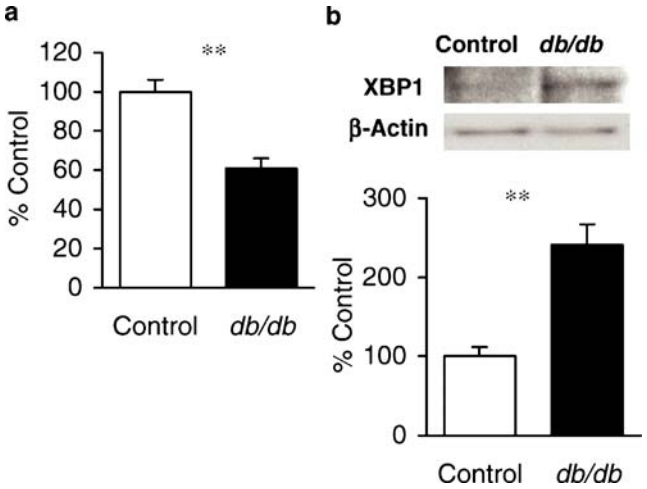

Fig. 7 Altered XBP1 splicing in islets of diabetic mice. **a** RNA extracted from islets isolated from lean control and diabetic *db*/*db* mice was reverse transcribed. *Xbp1* cDNA was amplified by PCR and digested with PST1, which cuts unprocessed *Xbp1* cDNA into fragments. Processed (activated) *Xbp1* cDNA lacks the restriction site and remains intact. Processed (intact) and unprocessed (cut) *Xbp1* was quantified by densitometry. The value obtained for cut *Xbp1* was expressed as a ratio of the total (processed + unprocessed) *Xbp1* mRNA levels for each animal. These ratios are expressed as a percentage of the ratio in control islets. Results are means±SEM determined from five mice per group. **b** Representative western blot comparing the expression of activated XBP1 protein (54 kDa) in islets isolated from control and diabetic *db*/*db* mice. β-actin is shown as a control of protein loading. Bands were quantitated by densitometry and the ratio of XBP1/β-actin expressed as a percentage of control values. Values are means±SEM determined from five mice per group. $^{**}p<0.01$

diabetic subjects, a finding consistent with previous studies [34], and was unaltered in islets of type 2 diabetes subjects (not shown).

Discussion

Because ER stress is sufficient to induce both beta cell death [12–15] and peripheral insulin resistance [35], its relevance to type 2 diabetes has been extensively canvassed. However, it has not yet been established whether ER stress actually occurs in type 2 diabetes or whether it makes a necessary contribution to the development of the disease. Our study addresses both of these shortfalls. First, we show a broad-based ER stress response induced in palmitate-treated MIN6 cells, an in vitro model of beta cell dysfunction. Second, we show activation of genes involved in ER stress, both in islets from *db*/*db* mice and from human type 2 diabetes subjects. Third, by demonstrating that overproduction of the ER chaperone HSPA5 partially protects against the effects of palmitate, we provide the first evidence that ER stress is required for beta cell lipoapoptosis, and by extension for the development of type 2 diabetes.

A partial requirement for ER stress has been previously described for cytokine-mediated beta cell apoptosis, involving nitric oxide generation, depletion of ER Ca^{2+} stores and, ultimately, induction of DDIT3 [17, 36]. In contrast, the slower development of beta cell destruction in type 2 diabetes suggests a more complex aetiology. Nevertheless, it is noteworthy that ER stress appears sufficient to cause beta cell apoptosis irrespective of the manner in which that stress is triggered. Thus, in Akita mice, a folding mutation in proinsulin is responsible [12]; in Wolfram syndrome ER Ca^{2+} handling is compromised [37]; whereas Walcott–Rallison syndrome involves a defect in the gene encoding EIF2AK3 [38]. These and the transgenic animal models of *Eif2ak3* deletion [13] or ablated EIF2A phosphorylation [14] all result in loss of beta cell mass and pathologies akin to type 2 diabetes. Thus, ER stress is an attractive mechanism for integrating the diverse inputs of a multifactorial disease such as type 2 diabetes and channelling them into a common, defining pathology.

Type 2 diabetes is associated with obesity and elevations in circulating fatty acids. Subtle perturbations in the capacity of pancreatic beta cells to cope with this enhanced fatty acid supply are therefore hypothesised to contribute to the onset of the disease [5, 6, 22]. In vitro, chronic fatty acid treatment of beta cells is sufficient to recapitulate both the secretory defects and apoptosis observed in type 2 diabetes [6, 18, 19]. In vivo, and over the longer term, adaptive changes might tend to protect the beta cell from chronic lipid exposure, except perhaps in the presence of predisposing defects in lipid handling or of the contribution of an additional parameter such as hyperglycaemia. Nevertheless, the in vitro models have helped define the downstream consequences of those predisposing metabolic alterations. Indeed, we show here that an extensive ER stress response must be included amongst those consequences. Importantly, we have also linked ER stress selectively to lipoapoptosis. This is based on the observation that the saturated fatty acid palmitate, but not the unsaturated fatty acid oleate, induced genes involved in ER stress and triggered apoptosis. Most importantly, lipotoxicity was significantly reduced by overproduction of HSPA5, the protein chaperone and key regulator of the UPR. This provides definitive evidence that ER stress is actually required for mediating beta cell apoptosis in response to fatty acid, thus suggesting a causal link between ER stress and aspects of beta cell dysfunction that are relevant to type 2 diabetes.

The differential cytotoxicity of saturated and unsaturated fatty acids has been extensively reported using beta cells [6, 23–26], although not in one study, where both oleate and palmitate triggered ER stress in INS-1E cells [17]. This discrepancy might relate to dosage effects since we employed fatty acids pre-coupled to BSA in a 3:1 ratio, within

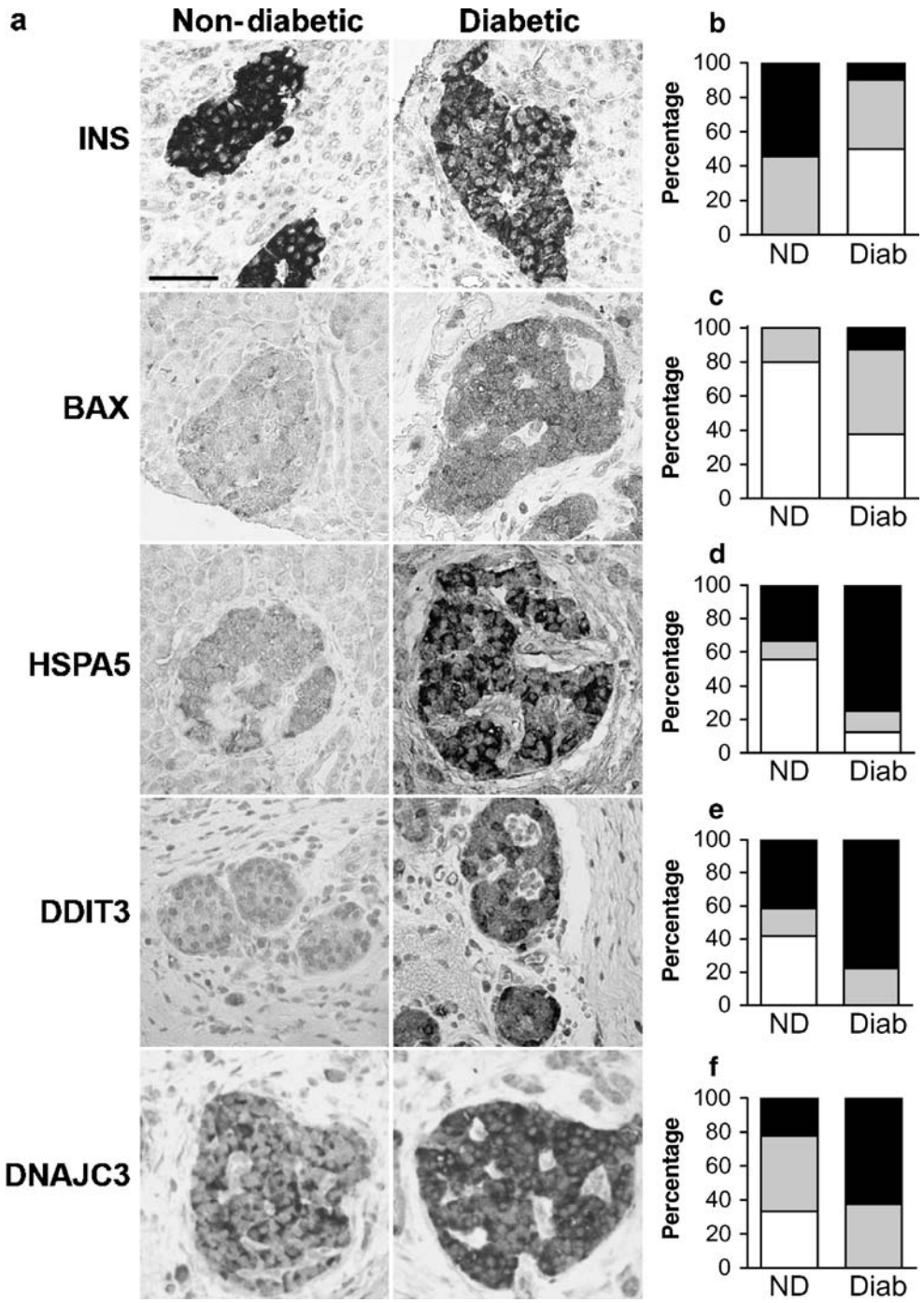

Fig. 8 **a** Increased BAX, HSPA5, DDIT3 and DNAJC3 staining and reduced insulin staining in islets from human type 2 diabetes subjects. Sections of pancreas arrays were immunostained for insulin, BAX, HSPA5, DDIT3 and DNAJC3, and staining intensity of islets scored. Representative images of immunostaining in islets from non-diabetic and type 2 diabetes subjects are shown for each protein. Magnification bar=50 μm. Immunostaining of islets for each subject was graded (**b**–**f**) as low (*open bars*), moderate (*grey bars*) or high (*dark bars*) intensity and expressed as a percentage of total normal and diabetes subjects, respectively, for insulin (**b**), BAX (**c**), HSPA5 (**d**), DDIT3 (**e**), DNAJC3 (**f**). *ND* non-diabetic subjects; *Diab* diabetic subjects

the physiologically elevated concentration range, as opposed to a concentrated fatty acid bolus dissolved in ethanol. Although other workers have more recently demonstrated selectivity for palmitate vs oleate in triggering ER stress and beta cell apoptosis [16], they have reported neither activation of all three ER stress sensors, nor a widespread elevation of UPR gene products. This might reflect subtle but important differences in experimental design. MIN6 cells are more highly differentiated than the rat INS-1 cells employed in the previous paper. Moreover, Karaskov et al. used serum-starved cells, a higher molar ratio of fatty acid to BSA (>6:1) and conducted their experiments at an elevated glucose concentration, known to exacerbate lipotoxicity [39, 40]. Under their conditions other apoptotic pathways might outstrip ER stress and perhaps curtail its full development. We employed a milder but longer exposure to fatty acids as more representative of chronic in vivo stress. We believe this approach is validated by the generally good concordance between our cell-based studies and the *db*/*db* islet data.

Table 2 Changes in mean intensity of immunostaining in islets of type 2 diabetes subjects compared with non-diabetic subjects

Gene	Non-diabetic subjects (*n*)	Diabetic subjects (*n*)	*p* value
INS	2.5±0.2 (11)	1.6±0.2 (10)	<0.01
BAX	1.1±0.2 (10)	1.8±0.3 (8)	<0.05
HSPA5	1.8±0.3 (9)	2.7±0.2 (9)	<0.05
DDIT3	2.0±0.3 (12)	2.8±0.1 (9)	<0.05
DNAJC3	1.9±0.3 (9)	2.6±0.2 (8)	<0.05

Islet immunostaining was graded and an immunoscore calculated by assigning a scale of 1 (low), 2 (moderate) or 3 (high) to staining intensity for each subject. Data are means±SEM determined from the number of subjects shown in parentheses.

The triggering of ER stress by palmitate is probably not due to nitric oxide generation, which is not apparent in pure beta cell populations such as MIN6 cells [19, 23]. Neither is deposition of the saturated triacylglycerol, tripalmitin, in ER [41] likely to be relevant, since, at the relatively low palmitate/BSA ratios used here, the increase in triacylglycerol mass observed in MIN6 cells is due primarily to the incorporation of unsaturated fatty acid side-chains, as the increase in triacylglycerol levels was abolished by desaturase inhibitors [19]. Increased formation of ceramide is a possibility since this is implicated in beta cell lipotoxicity [5, 6], and in other cell-types ceramide has been shown to perturb ER Ca^{2+} homeostasis [42].

Interestingly, islets of diabetic *db*/*db* mice also display abnormalities in ER Ca^{2+} mobilisation [43]. Because protein folding is critically dependent on ER Ca^{2+} content, these previously demonstrated abnormalities might be linked to ER stress which, as we show here for the first time, is also a characteristic of the *db*/*db* model. In general, the induction of individual UPR genes was more pronounced in *db*/*db* islets than in palmitate-treated MIN6 cells. This is not surprising given the vastly different time frames involved, and because beta cells in the animal model would have been exposed to elevations in blood glucose as well as circulating fatty acids. Indeed, an enhancement of XBP1 splicing was recently demonstrated in islets exposed to high glucose for 24 h in vitro [44]. This is consistent with our evidence of a prolonged activation of the IRE1/XBP1 arm both in *db*/*db* islets and fatty-acid-treated MIN6 cells. In contrast, ATF6 cleavage was transient in the cell system and was not observed at all in the animal model (results not shown), suggesting that this arm is either shorter lived than XBP1 signalling [45] or that the ATF6 cleavage product is rapidly degraded [46]. On the other hand, DDIT3 induction was more modest in the islets of *db*/*db* mice than in the cellular model, but this is still likely to be of functional relevance over a period of months.

A key aspect of our work is the indication that ER stress actually occurs in beta cells derived from human subjects with type 2 diabetes. Because this depended on immunohistochemical analysis of archival pathology sections, it was not possible to investigate splicing of *XBP1* mRNA, a definitive marker of ER stress. However, we provide evidence of induction of both HSPA5 and DNAJC3, which are predominately regulated in an ATF6- and XBP1-dependent manner, and hence are secondary to ER stress [28, 29]. Finally, we suggest that DDIT3 is also upregulated in human type 2 diabetes. This not only supports the presence of ER stress but also provides a potential mechanistic explanation for the apoptosis that appears to underlie the reductions in beta cell mass that contribute to type 2 diabetes in humans [2]. As with BAX, however, increased production of DDIT3 is not always sufficient to trigger cell death, although upregulation is indicative of an enhanced apoptotic potential [47, 48]. Therefore, further work is needed to evaluate the contribution of DDIT3 to beta cell lipotoxicity, compared with other pro-apoptotic pathways triggered by ER stress, such as MAPK8 and caspase-12. Nevertheless, our studies provide strong evidence that ER stress may be a crucial factor contributing to beta cell apoptosis in the pathogenesis of type 2 diabetes. Accordingly, increasing the chaperone capacity of the ER represents a potential therapeutic approach for preventing the onset of the disease.

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Duality of interest The authors declare that there is no duality of interest.

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