Diazoxide-induced β -cell rest reduces endoplasmic reticulum stress in lipotoxic β -cells

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Abstract

Elevated levels of glucose and lipids are characteristics of individuals with type 2 diabetes mellitus (T2DM). The enhanced nutrient levels have been connected with deterioration of β -cell function and impaired insulin secretion observed in these individuals. A strategy to improve β -cell function in individuals with T2DM has been intermittent administration of K_{ATP} channel openers. After such treatment, both the magnitude and kinetics of insulin secretion are markedly improved. In an attempt to further delineate mechanisms of how openers of K_{ATP} channels improve β -cell function, the effects of diazoxide on markers of endoplasmic reticulum (ER) stress was determined in β -cells exposed to the fatty acid palmitate. The eukaryotic translation

factor 2-alpha kinase 3 (EIF2AK3; also known as PERK) and endoplasmic reticulum to nucleus signaling 1 (ERN1; also known as IRE1) pathways, but not the activating transcription factor (ATF6) pathway of the unfolded protein response, are activated in such lipotoxic β -cells. Inclusion of diazoxide during culture attenuated activation of the EIF2AK3 pathway but not the ERN1 pathway. This attenuation was associated with reduced levels of DNA-damage inducible transcript 3 (DDIT3; also known as CHOP) and β -cell apoptosis was decreased. It is concluded that reduction of ER stress may be a mechanism by which diazoxide improves β -cell function.

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Introduction

Individuals with type 2 diabetes mellitus (T2DM) have impaired β -cell function, which leads to reduced or absent first phase and curtailment of the second phase in postprandial blood insulin levels (Porte & Kahn 2001). In addition, the normal rhythmic variations in blood insulin levels are replaced by irregular or lost insulin oscillations (Lang *et al.* 1981). Reduced islet insulin content is observed over time in these individuals (Rastogi *et al.* 1973, Marchetti *et al.* 2004), which has been attributed both to decreased β -cell granulation and reduced number of β -cells (Butler *et al.* 2003, Marchetti *et al.* 2004). Since insulin is the only glucose and lipid-lowering principal, inadequate insulin release is closely related to the elevated levels of circulating glucose and fatty acids observed in T2DM (Dimitriadis *et al.* 2004). Indeed, sustained increase in circulating free fatty acid levels has been connected with impaired insulin secretion in individuals predisposed to develop T2DM (Kashyap *et al.* 2003), where the altered insulin release kinetics (Lang *et al.* 1981, O'Rahilly *et al.* 1988) plays an important role by decreasing the ability of the hormone to exert its glucose and fatty acid-lowering effects (Paolisso *et al.* 1988). In an attempt to ameliorate the secretory

defects, diazoxide was administered to individuals with T2DM for 5 days (Greenwood *et al.* 1976). The drug hyperpolarizes the plasma membrane via opening of the ATP-sensitive K^+ channels leading to cessation of Ca^{2+} influx and insulin granular exocytosis leading to β -cell rest (Petit & Loubatieres-Mariani 1992, Seino *et al.* 1997). When insulin secretion was stimulated in these individuals after discontinuing the diazoxide treatment, a substantial increase in hormonal release was observed (Greenwood *et al.* 1976). Since the compound inhibits secretion, the beneficial effects were linked to reggranulation of the β -cells. Indeed, improved first- and second-phase insulin secretion as well as restored pulsatile insulin release has been achieved in individuals with T2DM after β -cell rest (Laedtke *et al.* 2000). Given the positive effects of diazoxide-induced β -cell rest in T2DM, mechanisms for how the compound exerts its positive effect have been investigated. In the present study, we wanted to explore whether induction of endoplasmic reticulum (ER) stress (Wu & Kaufman 2006), which has been observed in β -cells exposed to elevated levels of glucose and fatty acids (Kharroubi *et al.* 2004, Wang *et al.* 2005, Karaskov *et al.* 2006, Laybutt *et al.* 2007), was affected by diazoxide. We hypothesized that β -cell rest induced by diazoxide involves

attenuation of ER stress. To address the hypothesis, we investigated how diazoxide affected the different signaling pathways aiming at restoring the balance of the ER in β -cells exposed to the fatty acid palmitate.

Materials and Methods

Cell culture and islet isolation

All standard cell culture media and additives were obtained from Invitrogen. Rat insulinoma (INS-1E) cells (a kind gift from Dr Pierre Maechler, Geneva University) were maintained in RPMI 1640 (11 mM glucose) media supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, 100 μ g/ml streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM HEPES, and 55 μ M β -mercaptoethanol (all from Invitrogen) at 37 °C and 5% CO₂. All experiments with INS-1E cells were performed between passages 60 and 90. During palmitate exposure, the FBS concentration in the INS-1E cell culture medium was reduced to 1% (Kharroubi *et al.* 2004, Cnop *et al.* 2007) and 0.5% fatty acid-free BSA (Boehringer Mannheim GmbH).

Mouse insulinoma (MIN6) cells (a kind gift from Prof. Jun-ichi Miyazaki, Osaka University) were maintained in Dulbecco's Modified Eagle medium (DMEM) (25 mM glucose) supplemented with 15% FBS, 100 units/ml penicillin, 100 μ g/ml streptomycin, and 55 μ M β -mercaptoethanol at 37 °C and 5% CO₂. All experiments with MIN6 cells were performed between passages 21 and 28. During palmitate exposure, the FBS concentration in the MIN6 cell culture medium was kept at 15% (Laybutt *et al.* 2007) and 0.5% fatty acid-free BSA was added.

Islets were isolated, by collagenase digestion of pancreata, from male Wistar rats (B&K, Sollentuna, Sweden) weighing 250 g. After isolation, the islets were cultured for 24 h in RPMI 1640 (11 mM glucose) media supplemented with 10% FBS, 100 units/ml penicillin, and 100 μ g/ml streptomycin at 37 °C and 5% CO₂. During palmitate exposure, the FBS concentration in the islet cell culture medium was reduced to 0% and 0.5% fatty acid-free BSA was added. The procedures involving animals were in conformity with national and international laws for the care and use of laboratory animals and were approved by the local animal ethical committee.

Palmitate medium and cell/islet treatment

Culture medium containing palmitate (Sigma P-9767) was prepared by dissolving the fatty acid in 50% ethanol to a final concentration of 100 mM. The stock solution was then diluted in culture medium to a final concentration of 0.5 mM and allowed to complex with 0.5% BSA for 30 min at 37 °C (Karaskov *et al.* 2006). Final ethanol concentration (0.25%) was added to control cells. The cells were cultured to 80–85% confluence and stimulated with palmitate in the absence or presence of 100 μ M diazoxide (Sigma D-9035) for 0, 2, 4, 8, 12,

24, or 48 h. Islets precultured for 24 h were transferred to new culture medium with or without 0.5 mM palmitate. Some islets that were exposed to palmitate also received 100 μ M diazoxide. Islets were stimulated for 48 h.

Insulin secretion and insulin content measurements

Accumulated insulin secretion from INS-1E and MIN6 cells was determined by collecting culture media at the end of the culture period for the determination of released insulin. Glucose-stimulated insulin secretion (GSIS) was measured in INS-1E and MIN6 cells after culture by incubating the cells for 60 min at 37 °C with culture medium containing 2 mM glucose. Subsequently, the medium was changed to a buffer consisting of 2 mM glucose, 125 mM NaCl, 5.9 mM KCl, 1.2 mM MgCl₂, 1.3 mM CaCl₂, and 25 mM HEPES, titrated to pH 7.4 with NaOH, and supplemented with 1 mg/ml BSA (fraction V; Boehringer Mannheim GmbH). The cells were kept in the medium for 30 min at 37 °C before the medium was changed again to the same type of buffer containing either 2 or 20 mM glucose. The cells were incubated at 37 °C for 30 min. After incubation, aliquots of the buffer were taken for later determination of released insulin. The cells were subsequently washed with a BSA-free buffer and lysed in insulin release buffer supplemented with 0.1% Triton X-100 and 25 mM NaOH but without BSA. Samples were taken for the determination of insulin content, DNA content, and protein content (DC protein assay, Bio-Rad). Medium insulin, released insulin, and insulin content were determined after appropriate dilutions with ELISA, as described previously (Bergsten & Hellman 1993). Insulin release and content data were normalized to total protein measurements obtained by the DC protein assay.

Apoptosis measurements

Apoptosis in INS-1E and MIN6 cells was assayed by the cell death detection kit ELISA^{PLUS} (Roche Diagnostics), according to the manufacturer's instructions. The ELISA measures cytoplasmic oligonucleosomes that increase after apoptosis-associated DNA degradation. The apoptosis measurements were related to the DNA content obtained in separate experiments by lysing cells in Milli-Q water and then measuring the absorption at 260 nm.

Protein measurements of ER stress response markers

Protein levels of ER stress response markers were determined by western blotting. Samples for western blotting were prepared from INS-1E and MIN6 cells or from pancreatic islets by washing the cells/islets twice with PBS followed by lysing the cells/islets on ice with a buffer composed of 150 mM NaCl, 50 mM Trizma base, 1% Triton X-100, 0.25% Na-deoxycholate, 1 mM Na₃VO₄, 2 mM EGTA, and a protease inhibitory cocktail (Sigma P-8340) for 15 min. After lysis, the preparations were collected and centrifuged at 12 000 g for 10 min at 4 °C. The supernatants were transferred to new tubes and the total

protein concentration was determined by the DC protein assay, according to the manufacturer's instructions. Samples were mixed with SDS-PAGE sample buffer containing Tris-HCl (pH 6-8), SDS, glycerol, and dithiothreitol, and boiled for 5 min. The samples (20 µg per well) were then subjected to SDS-PAGE. After electrophoresis, the proteins were transferred onto polyvinylidene fluoride (PVDF) membrane. Immunoblot analyses were performed with antibodies toward p-EIF2A (also known as p-eIF2a) (Cell Signaling, Beverly, MA, USA), EIF2A (Cell Signaling), HSPA5 (also known as BiP/GRP78) (Abcam, Cambridge, UK), DDIT3 (Sigma), and ATF6 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Immunoreactive bands were imaged with Fluor-S MultiImager MAX (Bio-Rad) and quantified with Quantity One software (Bio-Rad). After imaging, the PVDF membranes were stained with Coomassie and later destained with 50% methanol. The blots were then scanned in a standard tabletop scanner and quantified with Quantity One software. The expression levels of each protein in each time point were normalized to the Coomassie-stained blot with the exception of p-EIF2A, which was normalized to total EIF2A content. In time-course experiments, the normalized value obtained at time 0 was set to 100% and the values at other time points were expressed as percentages thereof.

Transcript measurements of ER stress response genes

Transcript levels of ER stress response genes were determined by real-time PCR. Total mRNA was isolated from INS-1E cells by TRIzol (Invitrogen), according to the manufacturer's instructions, and reversely transcribed with SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). The real-time PCR was performed in 10 µl volume containing ~20 ng cDNA, 0.5 µM forward and reverse primers, and 5 µl DyNAmo Capillary SYBR Green qPCR kit (Finnzymes, Espoo, Finland). The primers used for amplification are shown in Table 1. PCR products were quantified fluorometrically using SYBR Green and normalized to the housekeeping gene β-actin and relative to the control (0 h). The following formula was used: target amount = 2^{-ΔΔC_t}, where ΔΔC_t = {(C_t(target gene sample) - C_t (β-actin sample)) - (C_t (control sample) - C_t (β-actin control))} (Livak & Schmittgen 2001).

Table 1 Primers used for real-time PCR

Target	Forward primer	Reverse primer
β-Actin	5'-TCTGTGTGGATTGGTGGCTC-3'	5'-GACTCATCGTACTCCTGCTTGCT-3'
ATF4	5'-GTTGGTCAGTGCCTCAGACA-3'	5'-CATTCGAAACAGAGCATCGA-3'
PPP1R15A	5'-GTCCATTTCTTGCTGTCTG-3'	5'-AAGGCGTGCCCATGCTCTGG-3'
DDIT3	5'-CCAGCAGAGGTCACAAGCAC-3'	5'-CGCACTGACCACTCTGTTTC-3'
HSPA5	5'-CCACCAGGATGCAGACATTG-3'	5'-AGGGCCTCCACTTCCATAGA-3'
HSP90B1	5'-TACTATGCCAGTCAGAAGAAAACG-3'	5'-CATCCTTTCTATCCTGTCTCCATA-3'
Spliced Xbp1 rat	5'-GAGTCCGCAGCAGGTG-3'	5'-GCGTCAGAATCCATGGGA-3'
Spliced Xbp1 mouse	5'-GAGTCCGCAGCAGGTG-3'	5'-GACTCTGACACTGTTGCCTC-3'

Total protein and insulin synthesis measurements

INS-1E cells were washed with PBS, starved for 30 min in RPMI 1640 medium lacking methionine and cysteine (SVA, Uppsala, Sweden), and labeled with Pro-mix [³⁵S]Met/Cys (120 µCi/ml; GE Healthcare). After 30 min, the cells were washed again with PBS, solubilized in 500 µl lysis buffer (1% Triton X-100 in PBS) for 15 min, scraped, and centrifuged for 10 min at 12 000 g, after which the total protein concentration was determined by the DC protein assay. To measure total protein synthesis, 2.5 ml Ultima Gold scintillation fluid (Chemical Instruments AB, Sollentuna, Sweden) were added to 50 µl lysate and radioactivity was determined by a liquid-scintillation spectrometer (Wallac System 1400; Perkin-Elmer, Boston, MA, USA). To measure insulin synthesis, insulin was precipitated from 400 µl lysate using insulin antibodies (Abcam) and Protein A Sepharose 4B Conjugate (Invitrogen), according to the manufacturer's instructions. The immunoprecipitate was washed twice with lysis buffer, once with PBS and water, and finally resuspended in 500 µl of 1 M acetic acid. After the addition of 2.5 ml scintillation fluid, radioactivity was determined by the liquid-scintillation spectrometer. In time-course experiments, the radioactivity values in each time point were normalized to total protein and the normalized value obtained at time 0 was set to 100% and the values at other time points were expressed as percentages thereof.

Data analysis

Results are presented as means ± S.E.M. Statistical significance between two conditions was analyzed by Student's *t*-test and between several groups using one-way ANOVA with Tukey's *post hoc* test. *P* < 0.05 was considered statistically significant.

Results

Diazoxide improves GSIS from lipotoxic β-cells by restoring insulin content and reducing apoptosis

Lipotoxic β-cells were obtained by culturing β-cell lines, INS-1E and MIN6, in the presence of palmitate for 24 or 48 h. Secretory activity was measured as accumulated insulin during the first 12 h

of the culture period. Culture of the cell lines under lipotoxic conditions caused enhanced accumulated insulin release, which was effectively inhibited by the introduction of diazoxide during culture (Table 2). Secretory activity was also determined by measuring insulin release at non-stimulatory and stimulatory glucose concentrations during 30 min at the end of the culture period. Whereas the relationship between insulin release at non-stimulatory and stimulatory glucose levels was impaired by palmitate in MIN6 cells, elevated levels of insulin release at non-stimulatory was observed in INS-1E cells (Fig. 1). When diazoxide was included during culture, the relationship between basal and stimulatory secretory levels was improved in both the cell lines primarily by enhancing the stimulatory secretory levels.

Insulin content and apoptosis was analyzed at the end of the culture period. When the cells were cultured under the lipotoxic conditions, insulin content was reduced in both the cell lines (Table 3). Inclusion of diazoxide during culture normalized the content. Apoptosis was elevated six- to ninefold when the cell lines were cultured in the presence of palmitate (Fig. 2). When diazoxide was included during culture, apoptosis induced by lipotoxicity was attenuated. Diazoxide alone did not affect apoptosis (Fig. 2).

Diazoxide attenuates ER stress in lipotoxic β-cells

To test the hypothesis whether improved function was associated with lowering of the ER stress response, the activation state of the different arms of the unfolded protein response (UPR) was determined in the lipotoxic β-cells cultured in the absence or presence of diazoxide.

The EIF2AK3 pathway of the ER stress response was investigated by measuring p-EIF2A, which showed significantly elevated levels of phosphorylation in both rat islets and cell lines compared with levels at time 0 (Fig. 3). In fact, increased levels were observed already after 2 h in dynamic measurements performed in the cell lines (Fig. 3B and C). When diazoxide was included during culture, the rise in p-EIF2A induced by the

Table 2 Insulin secretion from insulin-producing β-cells exposed to palmitate and diazoxide. INS-1E and MIN6 cells were cultured for 12 h in the absence or presence of 0.5 mM palmitate, together or not with 100 μM diazoxide. Insulin accumulation to the culture medium was assayed by ELISA and normalized to total protein. Results are presented as means ± S.E.M. of six to eight independent experiments

	Released insulin (pmol/12 h per μg protein)	
	INS-1E	MIN6
Treatment		
Untreated	0.7 ± 0.08	3.4 ± 0.1
Diazoxide	0.2 ± 0.02 [†]	1.5 ± 0.1 [†]
Palmitate	1.5 ± 0.1*	4.2 ± 0.1*
Palmitate + diazoxide	0.4 ± 0.02 [†]	2.9 ± 0.1 [†]

**P* < 0.05 denotes the effect of palmitate, and [†]*P* < 0.05 denotes the effect of diazoxide.

Figure 1 Glucose-stimulated insulin secretion (GSIS) from lipotoxic β-cells. (A) INS-1E and (B) MIN6 cells were cultured for 24 or 48 h respectively in the absence or presence of 0.5 mM palmitate and/or 100 μM diazoxide. After culture, GSIS was determined by incubating the cells at 2 (white bars) or 20 (black bars) mM glucose and insulin accumulation to the culture medium was assayed by ELISA and normalized to total protein. Results are presented as means ± S.E.M. of four to five independent experiments. **P* < 0.05 denotes palmitate effect, and #*P* < 0.05 denotes diazoxide effect.

lipotoxic conditions was reverted completely (MIN6 cells) or almost completely (islets and INS-1E cells). Diazoxide alone did not affect the phosphorylation of EIF2A (data not shown). Phosphorylation of EIF2A leads to general translational attenuation (Harding & Ron 2002). We therefore investigated whether total protein and insulin synthesis was affected in palmitate-treated cells. Indeed, after 4 h, total protein and insulin synthesis had decreased by ~70% (Fig. 4). After 24 h, the inhibition had decreased to 50% of the initial levels. When diazoxide was added to the culture medium, synthesis was further lowered in INS-1E exposed to palmitate. Diazoxide alone only caused a transient decrease in protein synthesis (data not shown).

Table 3 Insulin content in insulin-producing β-cells exposed to palmitate and diazoxide. INS-1E and MIN6 cells were cultured for 24 and 48 h respectively in the absence or presence of 0.5 mM palmitate, together or not with 100 μM diazoxide. After culture, the cells were lysed and cellular insulin content was assayed by ELISA and normalized to total protein. Results are presented as means ± S.E.M. of 12 independent experiments

	Insulin content (pmol/μg protein)	
	INS-1E	MIN6
Treatment		
Untreated	5.1 ± 0.2	2.6 ± 0.1
Diazoxide	5.6 ± 0.2	6.8 ± 0.2 [†]
Palmitate	3.9 ± 0.2*	1.2 ± 0.1*
Palmitate + diazoxide	5.9 ± 0.6 [†]	3.9 ± 0.2 [†]

**P* < 0.05 denotes the effect of palmitate, and [†]*P* < 0.05 denotes the effect of diazoxide.

If ER stress is not alleviated, DDIT3 expression levels are elevated, which may initiate apoptotic signaling pathways (Boyce & Yuan 2006, Cnop *et al.* 2007). In lipotoxic rat islets, INS-1E and MIN6 cells, DDIT3 protein levels were significantly increased at the end of the culture period (Fig. 5). The rise was actually evident already after 8 h in the cell lines (Fig. 5B and C). When diazoxide was added during culture, the increase in DDIT3 protein levels observed in the lipotoxic islets, INS-1E, and MIN6 cells was reverted totally (MIN6; Fig. 5C) or partially (islets, INS-1E; Fig. 5A and B). Exposure of islet, INS-1E, or MIN6 cells to diazoxide alone did not influence the levels of DDIT3 protein compared with untreated cells (data not shown). Enhanced expression of DDIT3 protein can be induced by different mechanisms, however. In support of a role of the EIF2AK3 signaling pathway and phosphorylation of EIF2A in enhanced DDIT3 expression in the lipotoxic β-cells, ATF4 and PPP1R15A (also known as GADD34) transcript levels were induced (Fig. 5D and E). When diazoxide was included in the culture medium, the elevation of *Atf4* and *Ppp1r15a* mRNAs were decreased.

The ERN1 pathway of the UPR was next investigated by measuring spliced forms of mRNA coding for *Xbp1* (Lee *et al.* 2002). In the lipotoxic rat islets and INS-1E and MIN6 cells, increased splicing of *Xbp1* was observed (Fig. 6). When the lipotoxic cells or islets were cultured in the presence of diazoxide, similar *Xbp1* splicing was observed (data not shown). Exposure to diazoxide alone did not induce splicing of *Xbp1* compared with control cells (data not shown).

Lastly, activity of the ATF6 pathway of the ER stress response was investigated by measuring the ratio between cleaved and uncleaved ATF6. A rise in processed ATF6 would lead to transcriptional activation of chaperone proteins (Lee *et al.* 2002). No induction of ATF6 cleavage was observed in the lipotoxic islets, INS-1E or MIN6 cells cultured in the absence or presence of diazoxide (data not shown). In support of the ATF6 pathway not being activated in the lipotoxic β-cells, the chaperone HSPA5 was neither up-regulated at the transcript

Figure 2 Apoptosis in lipotoxic β-cells. (A) INS-1E and (B) MIN6 cells were cultured for 24 or 48 h respectively in the absence (white bars) or presence (black bars) of 0.5 mM palmitate and/or 100 μM diazoxide. After culture, apoptosis was measured by ELISA. The amount of apoptosis in untreated cells was set to 100% and other values were expressed as percentage thereof. Results are presented as means ± S.E.M. of four to five independent experiments. **P* < 0.05 denotes palmitate effect, and #*P* < 0.05 denotes diazoxide effect.

level nor at the translational level in the islets, INS-1E, or MIN6 cells (data not shown). Inclusion of diazoxide during culture did not affect the ratio of cleaved and uncleaved ATF6 or protein levels of the chaperones (data not shown).

Discussion

In the present study, experimental evidence is provided supporting a role of attenuation of ER stress as a mechanism of improved β-cell function after diazoxide-induced β-cell rest. The concept of improving β-cell function by intermittent inhibition of insulin secretion, β-cell rest, in individuals with T2DM was attempted over 30 years ago in

Figure 3 Phosphorylation of EIF2A in lipotoxic β -cells. (A) Rat pancreatic islets were cultured for 48 h in the presence of 0.5 mM palmitate, together or not with 100 μ M diazoxide. (B) INS-1E and (C) MIN6 cells were cultured for the indicated time periods in the presence of 0.5 mM palmitate, together (filled squares) or not (open squares) with 100 μ M diazoxide. After culture, the cells and islets were lysed and subjected to SDS-PAGE. Cellular levels of phosphorylated and total EIF2A were determined by western blotting. The ratio of phosphorylated to total EIF2A was determined. Ratio at time 0 (INS-1E and MIN6) or under control conditions (islets) was set to 100% and other time points (INS-1E and MIN6) or the presence of palmitate (islets) were expressed as percentage thereof. Results are presented as means $\pm$ S.E.M. of three to four independent experiments. * $P < 0.05$ denotes the effect of palmitate compared with time 0, $^{\S}P < 0.05$ denotes effect of palmitate compared with untreated, and $^{\#}P < 0.05$ denotes the diazoxide effect at a given time point.

Figure 4 Total protein and insulin biosynthesis in lipotoxic β -cells. INS-1E cells were cultured for the indicated time periods in the presence of 0.5 mM palmitate, together (filled squares) or not (open squares) with 100 μ M diazoxide. At the end of the culture period, the cells were labeled with radioactive [35 S]methionine and cysteine. (A) After culture, total protein biosynthesis was measured by lysing the cells and determining radioactivity by a liquid-scintillation spectrometer. (B) To measure insulin synthesis, insulin was precipitated and radioactivity was determined by a liquid-scintillation spectrometer. The radioactive signal at time 0 was set to 100% and other time points were expressed as percentage thereof. Results are presented as means $\pm$ S.E.M. of five independent experiments. * $P < 0.05$ denotes the effect of palmitate and $^{\#}P < 0.05$ denotes the effect of diazoxide compared with time 0.

a study, where diazoxide was used to reduce the hormonal release (Greenwood *et al.* 1976). Diazoxide acts via opening of the K_{ATP} channels and includes repolarization, inhibited Ca^{2+} influx, and cessation of insulin granular exocytosis (Petit & Loubatieres-Mariani 1992, Seino *et al.* 1997).

When dissecting mechanisms for how diazoxide affects the β -cell in individuals with T2DM, different animal models of the disease and *in vitro* experimentation have been used. Similar to the individuals with T2DM, animal models like the diabetic Zucker fatty rat, the Goto Kakizaki (GK) rat, and the 90% pancreatectomized diabetic rat have perturbations in

Figure 5 Activation of the EIF2AK3 signaling pathway in lipotoxic β -cells. (A) Rat pancreatic islets were cultured for 48 h in the presence of 0.5 mM palmitate, together or not with 100 μ M diazoxide. (B, D, and E) INS-1E and (C) MIN6 cells were cultured for the indicated time periods in the presence of 0.5 mM palmitate, together (filled squares) or not (open squares) with 100 μ M diazoxide. After culture, total protein was isolated and subjected to SDS-PAGE where cellular levels of (A–C) DDIT3 were determined by western blotting. Also, total RNA was isolated reversely transcribed to cDNA and subjected to PCR with specific primers and transcript levels of (D) ATF4 and (E) PPP1R15A determined. Level at time 0 (INS-1E and MIN6) or under control conditions (islets) was set to 100% and other time points (INS-1E and MIN6) or the presence of palmitate (islets) were expressed as percentage thereof. Results are presented as means $\pm$ S.E.M. of three to four independent experiments. * $P < 0.05$ denotes the effect of palmitate compared with time 0, $^{\S}P < 0.05$ denotes the effect of palmitate compared with untreated, and $^{\#}P < 0.05$ denotes the diazoxide effect at a given time point.

Figure 6 Alternative splicing of the *Xbp1* mRNA in lipotoxic β -cells. (A) Rat pancreatic islets were cultured for 48 h in the absence or presence of 0.5 mM palmitate. (B) INS-1E (B) and (C) MIN6 cells were cultured for the indicated time periods at 0.5 mM palmitate. After culture, total RNA was isolated reversely transcribed to cDNA and subjected to PCR with specific primers for spliced *Xbp1*. The spliced *Xbp1* level at time 0 (INS-1E and MIN6) or under control conditions (islets) was set to 100% and other time points (INS-1E and MIN6) or the presence of palmitate (islets) were expressed as percentage thereof. Results are presented as means $\pm$ S.E.M. of three to four independent experiments. * $P < 0.05$ denotes the effect of palmitate compared with time 0.

SUR1), voltage-dependent calcium channel- $\alpha 1$, or potassium voltage-gated channel, Shab-related subfamily, member 1 (KCNB1; also known as Kv2.1) (Yoshikawa *et al.* 2004). By contrast, the expression levels of uncoupling protein 2 were reduced in diazoxide-treated islets, which was associated with augmented ATP/ADP ratio (Yoshikawa *et al.* 2004). The complex effects of diazoxide on the exocytotic machinery were further illustrated by the down-regulation of synaptosomal associated protein (SNAP25) and syntaxin (Ma *et al.* 2005) and the effects on interactions between syntaxin and ABCC8 (Ng *et al.* 2007). Some of these diazoxide-induced effects were corroborated when expression profiling was conducted on islets cultured at elevated glucose levels in the absence or presence of diazoxide (Ma *et al.* 2007). These effects of diazoxide on the cytoplasmic Ca^{2+} level and secretory activity made us investigate to what extent the effects also involved the ER stress response.

The results of the present study indicate that diazoxide-induced β -cell rest in lipotoxic cells is connected with reduced signaling via the EIF2AK3 pathway. By contrast, increased signaling via the ERN1 pathway, which was observed in lipotoxic β -cells in the present and previous studies (Kharroubi *et al.* 2004, Karaskov *et al.* 2006, Laybutt *et al.* 2007), was not affected by diazoxide. The EIF2AK3 and ERN1 pathways are parts of the cellular response aiming at restoring the balance of the ER, collectively called the UPR (Wu & Kaufman 2006). The EIF2AK3 pathway controls general protein translation and its activation leads to reduced translation. In the study, translational attenuation was observed in the lipotoxic β -cells. It can be speculated that the accentuation of this decline of synthesis in the lipotoxic cells by diazoxide may be a mechanism by which the hyperpolarizing agent exerts its β -cell protective effects. In the cells exposed to diazoxide alone, a transient decrease in insulin synthesis was observed, which was normalized after 24 h. The lack of the effect at the later time point is in agreement with a previous study, where no effect of the hyperpolarizing agent was found on insulin synthesis in the islets cultured for 3 days (Bjork *et al.* 1992). By contrast, another target of EIF2AK3 signaling is the proapoptotic protein DDIT3, which was reduced in the lipotoxic β -cells exposed to diazoxide. Elevated levels of glucose and fatty acids have separately and in combination been connected with the development of ER stress and enhanced DDIT3 expression in the β -cell (Kharroubi *et al.* 2004, Wang *et al.* 2005, Karaskov *et al.* 2006, Laybutt *et al.* 2007), although a direct causal link between the observed reduction in DDIT3 expression and attenuation of apoptosis has not been established. In support of a relationship between the events, enhanced apoptosis in the islets of individuals with T2DM was connected with enhanced DDIT3 expression (Huang *et al.* 2007, Laybutt *et al.* 2007). However, in another study, DDIT3 expression in the islets from the T2DM individuals was not elevated (Marchetti *et al.* 2007). The findings indicate that other mechanisms for palmitate-induced apoptosis, ER stress related or not, are operational in the pancreatic β -cell. Among such

mechanisms, tripalmitin formation has been proposed (Moffitt *et al.* 2005). Esterification of the fatty acid may not necessarily be β -cell toxic, however (Diakogiannaki *et al.* 2007).

In addition to the ability of diazoxide and related compounds to improve glucose tolerance in individuals with T2DM including obese subjects with T2DM (Guldstrand *et al.* 2002), the compound has been used to prolong the time of residual β -cell mass in individuals with T1DM (Bjork *et al.* 1996). In the latter individuals, cytokine-induced destruction is a prominent feature (Cnop *et al.* 2005). Since cytokines are potent inducers of ER stress (Kharroubi *et al.* 2004), the proposed action of diazoxide to attenuate ER stress may be an important aspect of the beneficial effects of the drug in this patient group.

In conclusion, we propose that diazoxide reduces β -cell apoptosis via attenuation of ER stress, which has been connected with T2DM. In addition, diazoxide-induced ER stress attenuation may be responsible for the positive effects of the drug in individuals with T1DM. The positive clinical experiences with β -cell rest for both patient groups call for continued efforts aiming at developing compounds inhibiting insulin secretion. Evaluation to what extent such compounds affect ER stress would be of great interest.

Declaration of interest

The authors declare that there is no conflict of interest.

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References

- Alemzadeh R & Tushaus K 2005 Diazoxide attenuates insulin secretion and hepatic lipogenesis in Zucker diabetic fatty rats. *Medical Science Monitor* **11** BR439–BR448.
- Bergsten P & Hellman B 1993 Glucose-induced amplitude regulation of pulsatile insulin secretion from individual pancreatic islets. *Diabetes* **42** 670–674.
- Bjork E, Kampe O, Andersson A & Karlsson FA 1992 Expression of the 64 kDa/glutamic acid decarboxylase rat islet cell autoantigen is influenced by the rate of insulin secretion. *Diabetologia* **35** 490–493.
- Bjork E, Berne C, Kampe O, Wibell L, Oskarsson P & Karlsson FA 1996 Diazoxide treatment at onset preserves residual insulin secretion in adults with autoimmune diabetes. *Diabetes* **45** 1427–1430.
- Bjorklund A, Ostenson CG & Grill VE 1997 Defective insulin secretion in the GK rat is not linked to excessive B-cell stimulation. *Pancreas* **14** 212–214.
- Bollheimer LC, Skelly RH, Chester MW, McGarry JD & Rhodes CJ 1998 Chronic exposure to free fatty acid reduces pancreatic beta cell insulin content by increasing basal insulin secretion that is not compensated for by a corresponding increase in proinsulin biosynthesis translation. *Journal of Clinical Investigation* **101** 1094–1101.
- Boyce M & Yuan J 2006 Cellular response to endoplasmic reticulum stress: a matter of life or death. *Cell Death and Differentiation* **13** 363–373.
- Butler AE, Janson J, Bonner-Weir S, Ritzel R, Rizza RA & Butler PC 2003 Beta-cell deficit and increased beta-cell apoptosis in humans with type 2 diabetes. *Diabetes* **52** 102–110.
- Cnop M, Welsh N, Jonas JC, Jorns A, Lenzen S & Eizirik DL 2005 Mechanisms of pancreatic beta-cell death in type 1 and type 2 diabetes: many differences, few similarities. *Diabetes* **54** (Suppl 2) S97–S107.
- Cnop M, Ladrerie L, Hekerman P, Ortis F, Cardozo AK, Dogusan Z, Flamez D, Boyce M, Yuan J & Eizirik DL 2007 Selective inhibition of eukaryotic translation initiation factor 2 α dephosphorylation potentiates fatty acid-induced endoplasmic reticulum stress and causes pancreatic beta-cell dysfunction and apoptosis. *Journal of Biological Chemistry* **282** 3989–3997.
- Diakogiannaki E, Dhayal S, Childs CE, Calder PC, Welters HJ & Morgan NG 2007 Mechanisms involved in the cytotoxic and cytoprotective actions of saturated versus monounsaturated long-chain fatty acids in pancreatic beta-cells. *Journal of Endocrinology* **194** 283–291.
- Dimitriadis G, Boutati E, Lambadiari V, Mitrou P, Maratou E, Brunel P & Raptis SA 2004 Restoration of early insulin secretion after a meal in type 2 diabetes: effects on lipid and glucose metabolism. *European Journal of Clinical Investigation* **34** 490–497.
- Efanova IB, Zaitsev SV, Zhivotovsky B, Kohler M, Efendic S, Orrenius S & Berggren PO 1998 Glucose and tolbutamide induce apoptosis in pancreatic beta-cells. A process dependent on intracellular Ca^{2+} concentration. *Journal of Biological Chemistry* **273** 33501–33507.
- El-Asaad W, Buteau J, Peyot ML, Nolan C, Roduit R, Hardy S, Joly E, Dbaibo G, Rosenberg L & Prentki M 2003 Saturated fatty acids synergize with elevated glucose to cause pancreatic beta-cell death. *Endocrinology* **144** 4154–4163.
- Goto Y, Suzuki K, Ono T, Sasaki M & Toyota T 1988 Development of diabetes in the non-obese NIDDM rat (GK rat). *Advances in Experimental Medicine and Biology* **246** 29–31.
- Greenwood RH, Mahler RF & Hales CN 1976 Improvement in insulin secretion in diabetes after diazoxide. *Lancet* **1** 444–447.
- Guldstrand M, Grill V, Bjorklund A, Lins PE & Adamson U 2002 Improved beta cell function after short-term treatment with diazoxide in obese subjects with type 2 diabetes. *Diabetes & Metabolism* **28** 448–456.
- Harding HP & Ron D 2002 Endoplasmic reticulum stress and the development of diabetes: a review. *Diabetes* **51** (Suppl 3) S455–S461.
- Hosokawa YA, Hosokawa H, Chen C & Leahy JL 1996 Mechanism of impaired glucose-potential insulin secretion in diabetic 90% pancreatectomy rats. Study using glucagonlike peptide-1 (7–37). *Journal of Clinical Investigation* **97** 180–186.
- Huang CJ, Lin CY, Haataja L, Gurlo T, Butler AE, Rizza RA & Butler PC 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.
- Karaskov E, Scott C, Zhang L, Teodoro T, Ravazzola M & Volchuk A 2006 Chronic palmitate but not oleate exposure induces endoplasmic reticulum stress, which may contribute to INS-1 pancreatic beta-cell apoptosis. *Endocrinology* **147** 3398–3407.
- Kashyap S, Belfort R, Gastaldelli A, Pratipanawatr T, Berria R, Pratipanawatr W, Bajaj M, Mandarino L, DeFronzo R & Cusi K 2003 A sustained increase in plasma free fatty acids impairs insulin secretion in nondiabetic subjects genetically predisposed to develop type 2 diabetes. *Diabetes* **52** 2461–2474.
- Kharroubi I, Ladrerie L, Cardozo AK, Dogusan Z, Cnop M & Eizirik DL 2004 Free fatty acids and cytokines induce pancreatic beta-cell apoptosis by different mechanisms: role of nuclear factor- κ B and endoplasmic reticulum stress. *Endocrinology* **145** 5087–5096.
- Laedtke T, Kjems L, Porksen N, Schmitz O, Veldhuis J, Kao PC & Butler PC 2000 Overnight inhibition of insulin secretion restores pulsatility and proinsulin/insulin ratio in type 2 diabetes. *American Journal of Physiology. Endocrinology and Metabolism* **279** E520–E528.
- Lang DA, Matthews DR, Burnett M & Turner RC 1981 Brief, irregular oscillations of basal plasma insulin and glucose concentrations in diabetic man. *Diabetes* **30** 435–439.

- Laybutt DR, Preston AM, Akerfeldt MC, Kench JG, Busch AK, Biankin AV & Biden TJ 2007 Endoplasmic reticulum stress contributes to beta cell apoptosis in type 2 diabetes. *Diabetologia* **50** 752–763.
- Leahy JL, Cooper HE, Deal DA & Weir GC 1986 Chronic hyperglycemia is associated with impaired glucose influence on insulin secretion. A study in normal rats using chronic *in vivo* glucose infusions. *Journal of Clinical Investigation* **77** 908–915.
- Leahy JL, Bumbalo LM & Chen C 1994 Diazoxide causes recovery of beta-cell glucose responsiveness in 90% pancreatectomized diabetic rats. *Diabetes* **43** 173–179.
- Lee K, Tirasophon W, Shen X, Michalak M, Prywes R, Okada T, Yoshida H, Mori K & Kaufman RJ 2002 IRE1-mediated unconventional mRNA splicing and S2P-mediated ATF6 cleavage merge to regulate XBP1 in signaling the unfolded protein response. *Genes and Development* **16** 452–466.
- Livak KJ & Schmittgen TD 2001 Analysis of relative gene expression data using real-time quantitative PCR and the $2(-\Delta\Delta C(T))$ method. *Methods* **25** 402–408.
- Lupi R, Dotta F, Marselli L, Del Guerra S, Masini M, Santangelo C, Patane G, Boggi U, Piro S, Anello M *et al.* 2002 Prolonged exposure to free fatty acids has cytostatic and pro-apoptotic effects on human pancreatic islets: evidence that beta-cell death is caspase mediated, partially dependent on ceramide pathway, and Bcl-2 regulated. *Diabetes* **51** 1437–1442.
- Ma Z, Portwood N, Foss A, Grill V & Bjorklund A 2005 Evidence that insulin secretion influences SNAP-25 through proteasomal activation. *Biochemical and Biophysical Research Communications* **329** 1118–1126.
- Ma Z, Portwood N, Brodin D, Grill V & Bjorklund A 2007 Effects of diazoxide on gene expression in rat pancreatic islets are largely linked to elevated glucose and potentially serve to enhance beta-cell sensitivity. *Diabetes* **56** 1095–1106.
- Maedler K, Spinas GA, Dyntar D, Moritz W, Kaiser N & Donath MY 2001 Distinct effects of saturated and monounsaturated fatty acids on beta-cell turnover and function. *Diabetes* **50** 69–76.
- Maedler K, Oberholzer J, Bucher P, Spinas GA & Donath MY 2003 Monounsaturated fatty acids prevent the deleterious effects of palmitate and high glucose on human pancreatic beta-cell turnover and function. *Diabetes* **52** 726–733.
- Marchetti P, Del Guerra S, Marselli L, Lupi R, Masini M, Pollera M, Bugliani M, Boggi U, Vistoli F, Mosca F *et al.* 2004 Pancreatic islets from type 2 diabetic patients have functional defects and increased apoptosis that are ameliorated by metformin. *Journal of Clinical Endocrinology and Metabolism* **89** 5535–5541.
- Marchetti P, Bugliani M, Lupi R, Marselli L, Masini M, Boggi U, Filippini F, Weir GC, Eizirik DL & Cnop M 2007 The endoplasmic reticulum in pancreatic beta cells of type 2 diabetes patients. *Diabetologia* **50** 2486–2494.
- Moffitt JH, Fielding BA, Evershed R, Berstan R, Currie JM & Clark A 2005 Adverse physicochemical properties of tripalmitin in beta cells lead to morphological changes and lipotoxicity *in vitro*. *Diabetologia* **48** 1819–1829.
- Ng B, Kang Y, Elias CL, He Y, Xie H, Hansen JB, Wahl P & Gaisano HY 2007 The actions of a novel potent islet beta-cell specific ATP-sensitive K^+ channel opener can be modulated by syntaxin-1A acting on sulfonylurea receptor 1. *Diabetes* **56** 2124–2134.
- O'Rahilly S, Turner RC & Matthews DR 1988 Impaired pulsatile secretion of insulin in relatives of patients with non-insulin-dependent diabetes. *New England Journal of Medicine* **318** 1225–1230.
- Ostenson CG, Chen J, Sheu L & Gaisano HY 2007 Effects of palmitate on insulin secretion and exocytotic proteins in islets of diabetic Goto-Kakizaki rats. *Pancreas* **34** 359–363.
- Paoiliso G, Sgambato S, Torella R, Varricchio M, Scheen A, D'Onofrio F & Lefebvre PJ 1988 Pulsatile insulin delivery is more efficient than continuous infusion in modulating islet cell function in normal subjects and patients with type 1 diabetes. *Journal of Clinical Endocrinology and Metabolism* **66** 1220–1226.
- Petit P & Loubatieres-Mariani MM 1992 Potassium channels of the insulin-secreting B cell. *Fundamental and Clinical Pharmacology* **6** 123–134.
- Porte D Jr & Kahn SE 2001 Beta-cell dysfunction and failure in type 2 diabetes: potential mechanisms. *Diabetes* **50** (Suppl 1) S160–S163.
- Prentki M, Joly E, El-Assaad W & Roduit R 2002 Malonyl-CoA signaling, lipid partitioning, and glucolipotoxicity: role in beta-cell adaptation and failure in the etiology of diabetes. *Diabetes* **51** (Suppl 3) S405–S413.
- Rastogi GK, Sinha MK & Dash RJ 1973 Insulin and proinsulin content of pancreases from diabetic and nondiabetic subjects. *Diabetes* **22** 804–807.
- Sako Y & Grill VE 1990 A 48-hour lipid infusion in the rat time-dependently inhibits glucose-induced insulin secretion and B cell oxidation through a process likely coupled to fatty acid oxidation. *Endocrinology* **127** 1580–1589.
- Seino S, Inagaki N, Namba N, Wang CH, Kotake K, Nagashima K, Miki T, Aguilar-Bryan L, Bryan J & Gonoi T 1997 Molecular basis of functional diversity of ATP-sensitive K^+ channels. *Japanese Journal of Physiology* **47** (Suppl 1) S3–S4.
- Song SH, Rhodes CJ, Veldhuis JD & Butler PC 2003 Diazoxide attenuates glucose-induced defects in first-phase insulin release and pulsatile insulin secretion in human islets. *Endocrinology* **144** 3399–3405.
- Stein DT, Stevenson BE, Chester MW, Basit M, Daniels MB, Turley SD & McGarry JD 1997 The insulinotropic potency of fatty acids is influenced profoundly by their chain length and degree of saturation. *Journal of Clinical Investigation* **100** 398–403.
- Unger RH 1995 Lipotoxicity in the pathogenesis of obesity-dependent NIDDM. Genetic and clinical implications. *Diabetes* **44** 863–870.
- Wang H, Kouri G & Wollheim CB 2005 ER stress and SREBP-1 activation are implicated in beta-cell glucolipotoxicity. *Journal of Cell Science* **118** 3905–3915.
- Wu J & Kaufman RJ 2006 From acute ER stress to physiological roles of the unfolded protein response. *Cell Death and Differentiation* **13** 374–384.
- Yoshikawa H, Ma Z, Bjorklund A & Grill V 2004 Short-term intermittent exposure to diazoxide improves functional performance of beta-cells in a high-glucose environment. *American Journal of Physiology. Endocrinology and Metabolism* **287** E1202–E1208.

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