Differential regulation of the endoplasmic reticulum stress response in pancreatic β -cells exposed to long-chain saturated and monounsaturated fatty acids

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Abstract

Exposure of pancreatic β -cells to long-chain fatty acids leads to the activation of some components of the endoplasmic reticulum (ER) stress pathway and this mechanism may underlie the ability of certain fatty acids to promote β -cell death. We have studied ER stress in BRIN-BD11 β -cells exposed to either the saturated fatty acid palmitate (C16:0) or the monounsaturated palmitoleate (C16:1). Palmitate (0.025–0.25 mM) induced the expression of various markers of the RNA-dependent protein kinase-like ER eukaryotic initiation factor 2 α (eIF2 α) kinase (PERK)-dependent pathway of ER stress (phospho-eIF2 α ; ATF4, activating transcription factor 4 and C/EBP homologous protein (CHOP-10)) although it failed to promote the expression of the ER chaperone GRP78. By contrast, palmitoleate did not induce any markers of the ER stress pathway even at concentrations as high as 1 mM. When palmitate and palmitoleate were added in combination, a marked

attenuation of the ER stress response occurred. Under these conditions, the levels of phospho-eIF2 α , ATF4 and CHOP-10 were reduced to less than those found in control cells. Palmitoleate also attenuated the ER stress response to the protein glycosylation inhibitor, tunicamycin, and improved the viability of the cells exposed to this agent. Exposure of the BRIN-BD11 cells to the protein phosphatase inhibitor, salubrinal, in the absence of fatty acids resulted in increased eIF2 α phosphorylation but this was abolished by co-incubation with palmitoleate. We conclude that saturated fatty acids activate components of the PERK-dependent ER stress pathway in β -cells, ultimately leading to increased apoptosis. This effect is antagonised by monounsaturates that may exert their anti-apoptotic actions by regulating the activity of one or more kinase enzymes involved in mediating the phosphorylation of eIF2 α .

Journal of Endocrinology (2008) **197**, 553–563

Introduction

The incidence of type 2 diabetes has sharply increased in Western society over recent years and this correlates with rising levels of obesity, implying that changing dietary habits and lifestyle may be closely associated with the development of type 2 diabetes. In particular, it has been proposed that the hyperlipidaemia which can accompany obesity may lead to an elevation in plasma levels of free fatty acids (Gordon 1960, Unger 1995, Sabin *et al.* 2007) which, in turn, contributes to the insulin resistance (Boden *et al.* 1994), impaired insulin secretion (Oprescu *et al.* 2007) and progressive decline in pancreatic β -cell mass seen in type 2 diabetes.

Fluctuations in the circulating levels of free fatty acids are probably important for the physiological regulation of insulin secretion in response to nutrients (Stein *et al.* 1996, Dobbins *et al.* 2002, Haber *et al.* 2003) but it is clear that a sustained elevation can be detrimental to pancreatic β -cells (Shimabukuro *et al.* 1998, Cnop *et al.* 2001, Piro *et al.* 2002). This response has been observed both *in vivo* and *in vitro* and is often termed

‘lipotoxicity’ (Newsholme *et al.* 2007, Poitout & Robertson 2008). Recent studies have suggested that the lipotoxic demise of the pancreatic β -cells may be related to a process of endoplasmic reticulum (ER) stress (Kharroubi *et al.* 2004, Karaskov *et al.* 2006, Eizirik *et al.* 2008) and that this culminates in enhanced cell loss by apoptosis. ER stress forms one component of a more general ‘integrated stress response’ displayed by mammalian cells (Ron & Walter 2007) which can subserve one of two apparently opposite functions. For example, the initial response to induction of ER stress culminates in the temporary inhibition of general protein synthesis which provides the cell with a window of opportunity during which it may recover from the stress stimulus and thereby restore normal homeostasis. However, if the stress stimulus is prolonged, then a secondary series of events is initiated which ultimately lead to the death of the cell by apoptosis (Ron 2002, Ron & Walter 2007). Thus, ER stress can be beneficial to a cell in the short term whereas it is detrimental if prolonged.

Increasing evidence implies that the activation of apoptosis in response to prolonged ER stress may underlie the death of

β -cells achieved during chronic exposure to saturated fatty acids (Kharroubi *et al.* 2004, Karaskov *et al.* 2006, Eizirik *et al.* 2008, Laybutt *et al.* 2007). Hence, it follows from this, that manoeuvres designed to relieve ER stress might have beneficial effects on the β -cell and might promote their survival in the face of elevated saturated fatty acid levels. This concept is important since it has emerged that β -cell lipotoxicity results from the activation of specific pro-apoptotic pathways that are differentially regulated by saturated and monounsaturated molecules. Thus, while long-chain saturated fatty acids such as palmitate (C16:0) and stearate (C18:0) are powerfully lipotoxic to pancreatic β -cells, the equivalent monounsaturated molecules (palmitoleate (C16:1) and oleate (C18:1)) are potently cytoprotective under *in vitro* conditions (Welters *et al.* 2004, Diakogiannaki *et al.* 2007, Dhayal *et al.* 2008). In the present work, we have investigated the possibility that this may reflect a differential activation of the integrated stress response by saturated and monounsaturated fatty acids. Furthermore, we have considered the specific possibility that the cytoprotective actions of monounsaturates might be mediated by direct antagonism of the integrated stress response, leading to the maintenance of β -cell viability under conditions which would otherwise favour their demise.

Materials and Methods

Cell culture

The rat pancreatic β -cell line BRIN-BD11 was used in all experiments. The cells were cultured in RPMI-1640 medium (Invitrogen) containing 11 mM glucose supplemented with 10% fetal calf serum (PAA Laboratories, Yeovil, Somerset, UK), 2 mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin (Invitrogen). The cells were grown in 75 cm² flasks at 37 °C and 5% CO₂.

Treatment of cells with fatty acids

A stock solution of 50 mM palmitate (Sigma) was prepared in 50% ethanol by heating to 70 °C. Palmitoleate (MP Biomedicals, Cambridge, UK) and methyl palmitoleate (Sigma) were prepared by mixing with 90% ethanol at room temperature to produce stock solutions of 90 mM. The fatty acid preparations were then bound to 10% fatty acid-free BSA (MP Biomedicals) by incubation for 1 h at 37 °C. The mixture was added to RPMI-1640 medium (containing 11 mM glucose) deprived of fetal calf serum. The final concentrations present in the cell environment were 1% for BSA and 0.5% for ethanol. The cells were seeded into six-well plates at densities of 10⁵ cells/well or 25 cm² flasks and incubated for 24 h in complete RPMI-1640 medium. The medium was then removed and replaced with relevant fatty acid/BSA complexes (in RPMI-1640 devoid of fetal calf

serum) for a further 18 h or as otherwise indicated. Controls received BSA and vehicle only.

Vital dye staining for estimation of viability

Following incubation, floating and attached cells were collected, centrifuged at 200 *g* for 5 min and resuspended in 250 μ l complete RPMI-1640 plus 250 μ l trypan blue (0.4% in PBS). Viable and dead cells were counted using a haemocytometer and the number of dead cells expressed as a percentage of the total for each condition.

Protein isolation: whole-cell extraction

Following incubation, cells were washed with ice-cold PBS and lysed with 0.5 ml lysis buffer (20 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% Triton-X supplemented with 10 μ l/ml protease inhibitor cocktail (Sigma) and 10 μ l/ml phosphatase inhibitor cocktail 1 and 2 (Sigma)) per 75 cm² flask, for 10 min. The flasks were scraped and the contents were transferred to microfuge tubes. Each sample was vortexed four times for 15 s and then centrifuged at 13 000 *g* for 10 min at 4 °C. The supernatant was retained for western blotting.

Protein isolation: nuclear and cytoplasmic protein extraction

The cells were cultured in 25 cm² flasks, then washed with ice-cold PBS and lysed for 15 min with a low-salt lysis buffer consisting of 20 mM HEPES, 1 mM EDTA, 1 mM MgCl₂, 10 mM KCl, 20% glycerol supplemented just before use with 10 μ l/ml protease inhibitor cocktail (Sigma), 10 μ l/ml phosphatase inhibitor cocktail 1 and 2 (Sigma), 1 mM dithiothreitol (DTT) and 0.5% Triton X-100. Cell lysates were transferred into microfuge tubes and centrifuged for 5 min at 2500 *g*. The supernatant constituted the cytoplasmic extract. The intact pellet was re-suspended in high-salt lysis buffer (400 mM NaCl, 20 mM HEPES, 1 mM EDTA, 1 mM MgCl₂, 10 mM KCl, 20% glycerol, supplemented just before use with 10 μ l/ml protease inhibitor cocktail (Sigma), 10 μ l/ml phosphatase inhibitor cocktails 1 and 2 (Sigma) and 1 mM DTT). After 30 min of incubation with simultaneous shaking, the cell lysates were centrifuged for 30 min at 20 000 *g*. The supernatant was retained as the nuclear extract. Both cytoplasmic and nuclear fraction was assessed for protein content using the bicinchoninic acid protein assay (Perbio Science, Northumberland, UK).

Western blotting

With 7.5 μ l 4 $\times$ lauryl dodecyl sulphate (LDS) sample buffer (Invitrogen) and 3 μ l β -mercaptoethanol (Sigma), 19.5 μ l sample (diluted in lysis buffer to yield equal

amounts of protein among samples—typically 50 μ g were loaded) were mixed. The mixtures were heated at 90 °C for 10 min and loaded onto the gel. The gel was run in MOPS SDS running buffer (Invitrogen) at 200 V for approximately 1 h. The transfer of proteins from the gel to PVDF membrane was performed at 80 V and 250 mA overnight. The membrane was blocked in a solution containing 5% fatty acid-free milk powder dissolved in TBS supplemented with Tween 20 (0.05%) for 1 h at room temperature.

The following primary antibodies were used to probe membranes: activating transcription factor 4 (ATF4), GRP78 (Santa Cruz), eukaryotic initiation factor 2 α (eIF2 α), p-eIF2 α (Cell Signalling), CHOP-10, β -actin, histone H3 (Sigma). The dilution used in all cases was 1:1000. Incubation lasted for 4 h at room temperature. The membrane was then washed thrice with Tween-TBS for at least 5 min each time. The appropriate secondary antibody (anti-rabbit or anti-mouse IgG) was diluted in Tween-TBS containing 1% powdered milk (1:15 000) and incubated with the blot for 1 h at room temperature.

For the detection of bands, the chemiluminescent system ECL Plus (Amersham) was used. The membrane was exposed to film (Kodak BioMax) and the intensity of bands was quantified using a Fluor-S Multi-imager analysis system (Quantity One software Biorad UK Ltd). On some occasions, the membranes were stripped (0.2 M glycine, 1% SDS and 0.1% Tween, pH 2) for 10 min at room temperature with slight agitation and were then re-probed.

Electron microscopy

BRIN-BD11 cells were treated with fatty acids after seeding into 25 cm² flasks. Cells were fixed in 2.5% glutaraldehyde (for 2 h), washed with 0.1 M sodium cacodylate buffer (three times) and postfixed in 1% osmium tetroxide for 2 h. The samples were dehydrated by sequential washings with increasing concentrations of ethanol (30, 50, 70 and 100%). Alcohol:resin mixtures of gradually increasing concentration (30, 50 and 70%) were used to infiltrate the cells. The samples were finally embedded in pure resin that was left to polymerise at 60–70 °C overnight. Ultra-thin sections were cut with a MicroStar diamond knife and attached to nickel grids. The samples were then negatively stained with a saturated solution of uranyl acetate in 70% ethanol followed by lead citrate. Images were collected on a JEOL 1200 EXII transmission electron microscope.

Data analysis

All experiments were performed on at least three separate occasions and triplicates of each condition were normally used in each experiment. The results are expressed as mean $\pm$ s.e.m. and the level of significance was calculated by using Student's *t*-test or ANOVA and was regarded as significant when $P < 0.05$.

Results

Electron microscopic analysis of BRIN-BD11 cells following treatment with fatty acids

In previous studies, we have shown that treatment of BRIN-BD11 cells with palmitate leads to an increase in cell death mediated by apoptosis and that this response is attenuated when palmitoleate is included in the incubation medium (Welters *et al.* 2004, 2006, Diakogiannaki *et al.* 2007). In confirmation of these observations, it was observed in the present work that exposure of the cells to 250 μ M palmitate for 18 h in the presence of BSA but no serum, resulted in a large loss of viability (control: $77 \pm 3\%$ live cells; palmitate: $21 \pm 2\%$; $P < 0.001$). By contrast, 250 μ M palmitoleate was not toxic and even improved the viability beyond that seen under control conditions ($90 \pm 3\%$ live cells). Moreover, it completely prevented the loss of viability caused by palmitate (250 μ M palmitate plus 250 μ M palmitoleate: $92 \pm 3\%$ live cells; $P < 0.001$ relative to palmitate alone).

In view of these data obtained in cell viability experiments, we next employed electron microscopy to examine the morphology of β -cells following exposure to fatty acids. Examination of the images revealed that both of the metabolisable fatty acids tested (palmitate and palmitoleate) caused alterations to the cellular morphology (Fig. 1). However, important qualitative differences were noted. Thus, palmitate-treated cells displayed the most obvious changes in morphology with the appearance of widely distended intracellular membranes that were not seen in control cells (Fig. 1B and C). This could be indicative of structural changes to the ER since ribosomes were frequently seen in association with these distended membranes in palmitate-treated cells (Fig. 1H and I). Many of the cells also displayed the characteristic features of apoptosis, including condensation and margination of nuclear chromatin. Cells exposed to palmitoleate for the same period of time displayed less evidence of grossly altered morphology although moderate distension of the ER was still seen (Fig. 1D). β -cells treated with both palmitate and palmitoleate also displayed abnormal morphological features although, surprisingly, the ER distension was less extensive than in cells exposed to palmitate alone (Fig. 1E). Moreover, such cells did not have condensed and margined chromatin, consistent with the improved viability associated with these incubation conditions (Welters *et al.* 2004, 2006, Diakogiannaki *et al.* 2007). Similar effects were noted following exposure of cells to the non-metabolisable unsaturated analogue, methyl palmitoleate, in the presence of palmitate although methyl palmitoleate itself failed to alter the cellular morphology (Fig. 1F and G).

Effects of fatty acids on CHOP-10 expression

To investigate whether the morphological changes to the ER observed in the electron micrographs were correlated with

Figure 1 Electron microscopic analysis of BRIN-BD11 cell morphology following exposure to fatty acids. BRIN-BD11 cells were incubated for 18 h in (A) the absence or (B–C) presence of 0.25 mM palmitate, (D) 0.25 mM palmitoleate, (E) 0.25 mM palmitate plus 0.25 mM palmitoleate, (F) 0.25 mM methyl palmitoleate or (G) 0.25 mM palmitate plus 0.25 mM methyl palmitoleate. The cells were fixed and processed for transmission electron microscopy. (H and I) The presence of ribosomes lining the distended ER membranes in palmitate-treated cells is revealed. Scale bars: A, C, D, F, G and I = 2 μ m, B and H = 500 nm and E = 5 μ m.

the activation of the ER stress pathways in fatty acid-treated cells, the expression of several markers of ER stress was studied. Three independent pathways of ER stress have been described and these are each regulated by different proteins localised to the ER membrane: PERK (RNA-dependent protein kinase-like endoplasmic reticulum eIF2 α kinase), (IRE1) inositol requiring 1 and ATF4 (Harding & Ron 2002). Upon activation, each of these controls a distinct pathway that serves as an adaptive mechanism to promote the restoration of normal ER function. However, if ER stress is persistent then apoptosis can be initiated (Ron & Walter 2007, Sundar Rajan *et al.* 2007) and this frequently involves the induction of CHOP-10, a protein whose expression is increased by activation of any, or all, of the three ER stress

pathways. Preliminary studies suggested that neither the IRE-1 nor the ATF6-dependent mechanism was involved in mediating fatty acid responses in BRIN-BD11 cells to a significant extent and, therefore, we focussed principally on the PERK-dependent pathway.

Culture of BRIN-BD11 cells in the presence of palmitate (0–0.15 mM) resulted in the induction of CHOP-10 in a dose-dependent manner (Fig. 2). By contrast, exposure of these cells to palmitoleate (even at concentrations as high as 1 mM) failed to promote CHOP-10 induction beyond that observed in control cells (Fig. 3). Controls consisted of cells cultured in serum-free medium plus BSA for 18 h and it is possible that these conditions may generate modest ER stress in the cells due to the absence of serum. This could, then,

Figure 2 Dose-dependent induction of CHOP-10 by palmitate treatment. (a) BRIN-BD11 cells were treated with increasing concentrations of palmitate (P-0.025 mM, 0.05 mM, 0.1 mM and 0.15 mM) for 18 h. Whole-cell protein was extracted and probed with a CHOP-10 antibody in order to determine the expression of CHOP-10 protein. β -Actin was used as a loading control. (b) The intensity of bands for CHOP-10 and β -actin were quantified densitometrically and the ratio of CHOP-10/ β -actin was calculated.

account for the low levels of CHOP-10 expression seen in the absence of fatty acids since incubation of cells in serum-replete medium was associated with minimal CHOP-10 expression (not shown). Tunicamycin was used as a positive control in these studies and, as expected, it caused a large rise in CHOP-10 expression, consistent with its ability to induce ER stress by promoting the accumulation of incorrectly folded proteins within this organelle. When the period of exposure to palmitoleate was increased to 42 h, CHOP-10

Figure 3 Effect of increasing concentrations of palmitoleate on the expression of CHOP-10. BRIN-BD11 cells were treated with increasing concentrations of palmitoleate (PO) (0.025 mM, 0.25 mM, 0.5 mM and 1 mM) for 18 h. Whole-cell protein was extracted and probed for CHOP-10 protein expression with a CHOP-10 antibody (upper panel). β -Actin was used as a loading control (lower panel). Tunicamycin (T; 1 μ g/ml) was used as a positive control.

induction remained at a minimal level whereas tunicamycin caused a very large increase under these conditions (not shown).

In the next series of experiments, palmitate and palmitoleate were used in combination and the expression of CHOP-10 monitored. In these studies, the palmitate concentration was maintained at a level that did not lead to extensive cell loss in order to facilitate the recovery of protein for analysis by western blotting. As shown in Fig. 4, CHOP-10 was still induced in palmitate-treated cells under these conditions but CHOP-10 induction was markedly attenuated when palmitoleate was introduced together with palmitate (Fig. 4). An identical inhibitory response was observed when methyl palmitoleate was substituted for palmitoleate (not presented) suggesting that metabolic activation of the monounsaturate is not required to mediate this effect.

CHOP-10 is known to be one of the target genes whose expression is increased following induction of ATF4 in the PERK-dependent pathway of ER stress (Oyadomari & Mori 2004). Thus, we next measured ATF4 induction in cells exposed to fatty acids (Fig. 5). As observed for CHOP-10, the levels of ATF4 protein were increased in palmitate-treated cells relative to controls, but this response was not seen in cells exposed to palmitoleate. Moreover, when these fatty acids were introduced in combination, ATF4 expression was attenuated relative to that seen with palmitate alone (Fig. 5).

Since ATF4 expression is regulated in response to changes in the phosphorylation state of eIF2 α this protein was also

Figure 4 Effect of fatty acids on CHOP-10 expression in BRIN-BD11 cells. (a) Western blots showing the expression of CHOP-10 and β -actin following treatment of BRIN-BD11 cells with 0.025 mM palmitate (P), 0.025 mM palmitoleate (PO), 0.025 mM palmitate plus 0.025 mM palmitoleate or 1 μ g/ml tunicamycin for 18 h. Whole-cell protein lysates were probed with CHOP-10 antibody (upper panel) while β -actin was used as a loading control (lower panel). (b) The intensity of the CHOP-10 and β -actin bands were scanned and expressed as a ratio. Results are expressed as means $\pm$ S.E.M. from three experiments. * P < 0.05 relative to control; ** P < 0.001 relative to palmitate alone; *** P < 0.001 relative to control.

Figure 5 Effect of fatty acids on ATF4 expression in BRIN-BD11 cells. (a) Western blot showing the expression of ATF4 following treatment of BRIN-BD11 cells with 0.025 mM palmitate (P), 0.025 mM palmitoleate (PO), 0.025 mM palmitate plus 0.025 mM palmitoleate or 1 μM thapsigargin (Th) for 18 h. The nuclear fraction was recovered and probed with an antibody against ATF4 protein (upper panel). Histone was used as a loading control (lower panel). (b) The intensity of the bands for ATF4 and histone was analysed by densitometry and the ratio of ATF4/histone calculated from three experiments. The presence of 0.25 mM palmitate significantly increased ATF4 expression (* $P<0.05$) whereas 0.025 mM palmitoleate (either alone or together with palmitate) caused a marked reduction in expression to a value below the control level (** $P<0.01$).

studied. Increased phosphorylation of eIF2α was detected in palmitate-treated cells but was not observed in response to palmitoleate. Furthermore, the palmitate-induced phosphorylation of eIF2α was inhibited by the presence of palmitoleate (Fig. 6), consistent with suppression of this arm of the ER stress response in cells exposed to the unsaturated fatty acid.

Initiation of ER stress is often accompanied by changes in the disposition and expression of the ER chaperone, GRP78. However, in BRIN-BD11 cells, the expression of this protein was below the level of detection under both control conditions and after incubation with fatty acids. By contrast, tunicamycin caused a marked up-regulation of GRP78 (Fig. 7) suggesting that the level of ER stress generated in response to this agent was much greater than that elicited by incubation with any of the fatty acids under our experimental conditions.

Effect of palmitoleate on ER stress induced by tunicamycin

Since the above results indicate that exposure of β-cells to palmitoleate for 18 h not only fails to induce ER stress but also minimises the activation of ER stress initiated by palmitate, it was of interest to establish whether the monounsaturate also attenuates the ER stress response in cells exposed to

Figure 6 Effect of fatty acids on the expression of p-eIF2α in BRIN-BD11 cells. (a) Western blot showing the expression of p-eIF2α in response to 0.025 mM palmitate (P), 0.025 mM palmitoleate (PO), 0.025 mM palmitate plus 0.025 mM palmitoleate (P + PO) and 1 μM thapsigargin (Th). Following exposure to the fatty acids for 6 h, cytoplasmic protein was extracted and probed for p-eIF2α expression (upper panel). Total eIF2α was used as a loading control (lower panel). (b) The intensity of the p-eIF2α and total eIF2α bands were scanned densitometrically and expressed as a ratio. Results are expressed as means ± s.e.m. from three experiments. * $P<0.05$ relative to control; ** $P<0.001$ relative to palmitate alone; *** $P<0.001$ relative to control.

tunicamycin. Therefore, cells were treated with tunicamycin in the absence or presence of palmitoleate and markers of the PERK-dependent ER stress response measured. These studies revealed that, even when present at a low concentration (25 μM), palmitoleate markedly reduced the expression of both CHOP-10 and ATF4 induced by tunicamycin (Fig. 8a). In the presence of a higher concentration of palmitoleate (0.25 mM) the effect was even more marked, although the reduction in expression of the markers was not completely returned to

Figure 7 Effect of fatty acids on the expression of GRP78 in BRIN-BD11 cells. BRIN-BD11 cells were treated with 0.025 mM palmitate (P), 0.025 mM palmitoleate (PO) or 0.025 mM palmitate plus 0.025 mM palmitoleate (P + PO) for 18 h with 1 μg/ml tunicamycin (T) used as positive control. GRP78 expression was studied by western blotting in whole-cell extracts (upper panel) while β-actin was used as a loading control.

Figure 8 Effect of palmitoleate on the expression of CHOP-10, ATF4 and phospho-eIF2 α induced by tunicamycin. (a) BRIN-BD11 cells were treated with 0, 25 μ M or 250 μ M palmitoleate (PO) in the presence or absence of 1 μ M/ml tunicamycin (T) for 18 h. The expression of ATF4 protein (upper panel) or CHOP-10 (lower panel) was measured by western blotting. β -Actin was used as a loading control. Similar results were obtained in three experiments. (b) Western blot showing the expression of p-eIF2 α in BRIN-BD11 cells treated with 250 μ M palmitoleate (PO) in the presence or absence of 1 μ M/ml tunicamycin (T) for 6 h. Cytoplasmic protein was extracted and probed for either p-eIF2 α expression (upper panel) or total eIF2 α (lower panel). Equivalent results were obtained in three experiments.

control levels. Palmitoleate also inhibited the phosphorylation of eIF2 α induced by tunicamycin in BRIN-BD11 cells (Fig. 8b). In accord with these results, the loss of cell viability associated with exposure to tunicamycin was significantly attenuated when palmitoleate was present in the incubation medium (Fig. 9).

Figure 9 Effect of palmitoleate on tunicamycin toxicity in BRIN-BD11 cells. BRIN-BD11 cells were treated with 1 μ M/ml tunicamycin (T) in the presence or absence of 0.25 mM or 0.025 mM palmitoleate (PO) for 18 h. Viability was estimated with vital dye staining. Results shown are means $\pm$ S.E.M. from three experiments. * P < 0.05 compared with 1 μ M/ml tunicamycin alone.

The effect of palmitoleate was examined also on the expression of GRP78 induced by tunicamycin. As shown in Figs 7 and 10, tunicamycin caused increased levels of GRP78. The presence of palmitoleate did not affect the expression of GRP78 caused by tunicamycin. This result is of particular importance because it represents the first marker of ER stress whose expression was not attenuated by palmitoleate.

Effects of palmitoleate and salubrinal on eIF2 α phosphorylation

Since we were able to detect a reduction in stimulus-dependent phosphorylation of eIF2 α in cells exposed to palmitoleate, we next examined the possibility that an upstream kinase may be regulated in fatty acid-treated cells. The ER-associated kinase, PERK, frequently fulfils this role in response to ER stress but, somewhat surprisingly, we were unable to detect changes in PERK phosphorylation (an index of PERK activation since the phosphorylation reaction is autocatalytic) in response to palmitate or palmitoleate in BRIN-BD11 cells under conditions when thapsigargin caused an increase in phosphorylation (not presented). An alternative strategy was therefore adopted by examining the effects of palmitoleate in the presence of a protein phosphatase (PP) inhibitor, salubrinal (Boyce *et al.* 2005). This agent is reported to act as a selective inhibitor of PP1 which mediates dephosphorylation of eIF2 α in the ER stress cascade. In accordance with this, exposure of BRIN-BD11 cells to 25 μ M salubrinal alone resulted in a net increase in the level of phospho-eIF2 α over 24 h (Fig. 11). This response was maintained for up to 72 h in the continued presence of salubrinal (not shown) and probably reflects the interruption of a constitutive cycle operating between the phospho- and native forms in unstimulated cells. Palmitoleate did not reproduce this response to salubrinal suggesting that it does not inhibit PP1 activity but, strikingly, the monounsaturate dramatically reduced the level of phospho-eIF2 α in cells exposed to salubrinal. This implies that palmitoleate may exert its influence by inhibiting the activity of a kinase responsible for phosphorylation of eIF2 α in BRIN-BD11 cells.

Figure 10 Effect of palmitoleate on tunicamycin-induced expression of GRP78 in BRIN-BD11 cells. The expression of GRP78 in response to tunicamycin in the presence or absence of a range of concentrations of palmitoleate (PO) was studied by western blotting in BRIN-BD11 cells. Cells were treated with 0, 25 μ M or 250 μ M palmitoleate in the presence or absence of 1 μ M/ml tunicamycin for 18 h.

Figure 11 Effect of palmitoleate on salubrinal-induced expression of p-eIF2 α in BRIN-BD11 cells. (a) BRIN-BD11 cells were treated with 0.25 mM palmitoleate (PO) in the presence or absence of 25 μ M salubrinal (sal) for 6 h. Cytoplasmic protein was extracted and probed for expression of p-eIF2 α with a p-eIF2 α -specific antibody (upper panel). Total eIF2 α was used as a loading control (lower panel). (b) The bands were scanned by densitometry and expressed as the ratio of p-eIF2 α to total eIF2 α . Results are means $\pm$ S.E.M. from three experiments. * $P < 0.01$ relative to control; ** $P < 0.01$ relative to palmitate alone.

Discussion

Exposure of pancreatic β -cells to elevated levels of free fatty acids has been shown to be detrimental to viability and function both *in vitro* (Zhou & Grill 1994, Cnop *et al.* 2001, Piro *et al.* 2002) and *in vivo* (Carpentier *et al.* 2000). However, the situation is complex since studies with isolated human islets, primary rat β -cells and various β -cell lines have demonstrated that certain long-chain saturated fatty acids have the greatest propensity to induce cytotoxicity, while shorter chain saturated molecules ($< C14$) and long-chain unsaturated species are much less damaging. Indeed, some unsaturated fatty acids are potently cytoprotective and can attenuate the loss of viability caused by their saturated counterparts (Maedler *et al.* 2003, Welters *et al.* 2004, Diakogiannaki *et al.* 2007, Dhayal *et al.* 2008). This implies that fatty acids exert differential effects according to their chain length and degree of unsaturation but the mechanisms that underlie these differences have not been defined. In the present work, we have employed the BRIN-BD11 cell line as a model system since this cell responds similarly to human β -cells in terms of its responses to saturated and unsaturated fatty acids (Maedler *et al.* 2003). Thus, although these cells are transformed they appear to retain the essential responses observed in primary β -cells.

The functional differences seen upon exposure of BRIN-BD11 β -cells to saturated (palmitate) and monounsaturated (palmitoleate) fatty acids are correlated, in part, with differences in cell morphology. In particular, electron microscopic analysis revealed that exposure of the cells to palmitate (C16:0) caused severe morphological abnormalities that were manifest principally as distended intracellular membrane structures. Similar observations were made recently by Moffitt *et al.* (2005) and Lai *et al.* (2008) in β -cells. Examination of the affected membranes revealed that these were frequently decorated with ribosomes and it seems likely, therefore, that the changes represent structural modifications to the ER. Most of the cells displayed such changes and, in some cases, the distended membranes occupied much of the intracellular space.

Exposure of cells to palmitoleate (C16:1) caused qualitatively similar changes to the intracellular architecture but, in this case, the extent of membrane distension was much less extensive. This suggests that the incorporation of mono-unsaturated fatty acids into membrane lipids is less destructive to membrane architecture than when saturated molecules are present. However, the most surprising result was observed when the two species of fatty acid were applied in combination. Under these conditions (when the total fatty acid concentration was double that achieved with either molecule alone), the extent of cell damage was no greater than that seen with palmitate alone. Indeed, examination of multiple images revealed that there was a diminution of the overall extent of membrane distension compared with palmitate alone.

The presence of potential morphological alterations to the ER prompted an examination of relevant biochemical markers which might indicate the development of ER stress during exposure to fatty acids. Such studies revealed that the transcription factors ATF4 and CHOP-10 were markedly induced in BRIN-BD11 cells exposed to palmitate, which is consistent with the operation of at least one arm of the ER stress pathway. In support of this, phosphorylation of the initiation factor, eIF2 α was also increased suggesting that activation of the PERK-dependent arm of ER stress had occurred. However, no consistent induction of the ER chaperone, GRP78, was observed; a result that confirms recent findings in β -cells (Karaskov *et al.* 2006, Lai *et al.* 2008) but was unexpected given the primary involvement of this protein in regulating protein folding during ER stress and in activation of the PERK-dependent pathway (Eizirik *et al.* 2008, Ron & Walter 2007). Thus, exposure of cells to palmitate caused morphological abnormalities that are consistent with altered ER disposition and led to induction of certain distal components of the ER stress pathway. However, full activation of the PERK-dependent ER stress response was not seen. This conclusion is also consistent with other recent findings of Karaskov *et al.* (2006) who noted that ER Ca^{2+} stores do not become depleted during fatty acid cytotoxicity in β -cells, although, in contrast to the present

work, these authors did observe evidence of PERK activation in palmitate-treated cells. When cells were treated with tunicamycin, an inhibitor of N-linked protein glycosylation in the ER, marked up-regulation of all of the components studied was observed (including GRP78, p-eIF2 α , ATF4 and CHOP-10). Thus, exposure of β -cells to palmitate appears to activate some components of the integrated stress response that is sufficient to promote cell loss by apoptosis. Changes in ER stress markers have recently been observed in the islets of patients with type 2 diabetes (Huang *et al.* 2007, Marchetti *et al.* 2007) suggesting that ER stress might contribute to the progressive loss of β -cells which occurs in this condition, although it is not yet clear whether this reflects exposure of β -cells to elevated levels of saturated fatty acids *in vivo*.

In BRIN-BD11 cells, the activation of components of the ER stress pathway by palmitate was largely confined to the PERK-dependent arm whereas other workers have observed significant activation of additional ER stress pathways (Kharroubi *et al.* 2004, Karaskov *et al.* 2006). The reasons for this difference are not clear but it is noteworthy that Lai *et al.* (2008) recently reported selective activation of the PERK-dependent pathway when INS-1 cells were exposed to low concentration of palmitate (Lai *et al.* 2008). Thus, it is possible that differences in the free fatty acid concentrations achieved (which are dramatically influenced by proteins present in the incubation medium) may influence the extent to which each pathway is activated and that the PERK arm responds to lower palmitate concentrations.

Exposure of BRIN-BD11 cells to palmitoleate for 18 h failed to cause the induction of any of the markers of ER stress investigated, thereby revealing a critical difference in the biochemical response of the cells to saturated and monounsaturated fatty acids. This result is consistent with other data obtained recently in additional pancreatic β -cell lines (Karaskov *et al.* 2006, Laybutt *et al.* 2007) and in hepatocytes (Wei *et al.* 2006). More surprisingly, however, we observed that, despite its failure to normalise the altered morphology of palmitate-treated cells, palmitoleate markedly suppressed the induction of ER stress markers in palmitate-treated cells. Thus, the phosphorylation of eIF2 α and the induction of both ATF4 and CHOP-10 were attenuated in cells treated with palmitate plus palmitoleate when compared with those exposed to palmitate alone. Since, however, this occurred in the absence of any alteration in GRP78 expression during exposure to fatty acids, this implies that the response to palmitoleate was not initiated by a change in GRP78 expression but that the monounsaturate intervenes at a more distal point in the pathway. In this context, it is interesting to note that Lai *et al.* (2008) have recently reported that altered expression of GRP78 does not modify the loss of viability seen in β -cells exposed to fatty acids.

The results also revealed, for the first time, that palmitoleate attenuated the activation of ER stress in cells exposed to tunicamycin, thereby demonstrating that the protective

response is not restricted only to the activation of ER stress by saturated fatty acids. Moreover, the loss of cell viability arising from the presence of tunicamycin was also inhibited by palmitoleate, confirming our earlier conclusion that the monounsaturate can regulate cell death induced by a variety of different stimuli in β -cells (Welters *et al.* 2004, Diakogiannaki *et al.* 2007).

In order to examine, in more detail, the mechanisms by which the PERK-dependent arm of ER stress is attenuated by palmitoleate, the phosphorylation of eIF2 α was studied. Phosphorylation of eIF2 α represents a key triggering event in the pathway and the reaction can be catalysed by any one of the several cellular kinases (Rutkowski & Kaufman 2003). As such, it represents a bifurcation point within the ER stress pathway at which other stimuli (some of which may act independently of ER stress) can intervene. Thus, activation of one or more of these alternative kinases can elicit the downstream responses associated with ER stress without necessarily inducing ER stress itself. The dephosphorylation of eIF2 α is catalysed by PP1 and this enzyme is a selective target for the drug, salubrinal, which inhibits de-phosphorylation of the initiation factor (Boyce *et al.* 2005). Therefore, in order to investigate the possible involvement of altered eIF2 α phosphorylation in mediating the actions of palmitoleate, the response to salubrinal was examined.

As reported recently in PC12 cells (Boyce *et al.* 2005) and in primary rodent β -cells (Cnop *et al.* 2007), exposure of BRIN-BD11 cells to salubrinal maintained eIF2 α in a phosphorylated state. However, when palmitoleate was added in the simultaneous presence of salubrinal, the extent of phosphorylation of eIF2 α was decreased. Since, under these conditions, the phosphatase activity was restrained by salubrinal, these data could be taken to indicate that palmitoleate acted to inhibit the activity of an eIF2 α kinase (rather than promoting an increase in phosphatase activity). It should also be acknowledged, however, that palmitoleate may have simply impaired the action of salubrinal, although this mechanism would not provide a satisfactory explanation for its capacity to attenuate eIF2 α phosphorylation in response to other stimuli. If palmitoleate does restrain the activity of an eIF2 α kinase, then this does not necessarily imply that its actions are mediated directly since intermediate steps could be involved.

In considering these issues, it is important to note the recent proposal of Yusta *et al.* (2006) that protein kinase A (PKA) may be a critical mediator of the anti-apoptotic effects of the GLP-1 receptor agonist exendin-4 in purified rat β -cells (Yusta *et al.* 2006). This effect is secondary to a rise in cAMP and the authors show that the ensuing activation of PKA regulates several components of the ER stress pathway in β -cells. However, we consider it unlikely that PKA plays a role in mediating the effects of palmitoleate reported here since this fatty acid does not increase cAMP levels in BRIN-BD11 cells (Welters *et al.* 2006).

Taken together, the present results reveal that some, though not all, components of the PERK-dependent arm of the integrated stress response are activated in BRIN-BD11 cells exposed to palmitate. By contrast, in cells treated with palmitoleate, the integrated stress response remains silent. Exposure of β -cells to both palmitate and palmitoleate leads to attenuation of the integrated stress pathway and a similar response is seen in cells incubated with tunicamycin. We propose that these effects may reflect the diminished activation of an eIF2 α kinase(s) in response to palmitoleate. As such, it seems possible that palmitoleate does not simply relieve ER stress at its point of initiation in β -cells (as evidenced by the failure of palmitoleate to block completely the morphological changes to the ER seen in palmitate-treated cells) but, rather, it intervenes to inhibit the effector arm of one of the pathways by which prolonged ER stress elicits an increase in apoptosis.

Acknowledgements

We thank Boehringer-Ingelheim, The European Foundation for Study of Diabetes and the Diabetes and Wellness Research Foundation for financial support of this project. Grateful thanks are also due to Roy Moate for assistance with electron microscopy. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Received in final form 19 March 2008

Accepted 20 March 2008

Made available online as an Accepted Preprint
20 March 2008