

Reduced endoplasmic reticulum (ER)-to-Golgi protein trafficking contributes to ER stress in lipotoxic mouse beta cells by promoting protein overload

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Received: 4 June 2009 / Accepted: 28 July 2009 / Published online: 29 August 2009
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Abstract

Aims/hypothesis Saturated fatty acids augment endoplasmic reticulum (ER) stress in pancreatic beta cells and this is implicated in the loss of beta cell mass that accompanies type 2 diabetes. However, the mechanisms underlying the induction of ER stress are unclear. Our aim was to establish whether saturated fatty acids cause defects in ER-to-Golgi protein trafficking, which may thereby contribute to ER stress via protein overload.

Methods Cells of the mouse insulinoma cell line MIN6 were transfected with temperature-sensitive vesicular stomatitis virus G protein (VSVG) tagged with green fluorescent protein to quantify the rate of ER-to-Golgi protein trafficking. I14 antibody, which detects only correctly folded VSVG, was employed to probe the folding environment of the ER. ER stress markers were monitored by western blotting.

Results Pretreatment with palmitate, but not oleate, significantly reduced the rate of ER-to-Golgi protein trafficking assessed using VSVG. This was not secondary to ER stress, since thapsigargin, which compromises chaperone function by depletion of ER calcium, markedly inhibited VSVG folding and promoted strong ER stress but only slightly reduced protein trafficking. Blockade of ER-to-Golgi protein trafficking with brefeldin A (BFA) was sufficient to trigger ER stress, but neither BFA nor palmitate compromised VSVG folding.

Conclusions/interpretation Reductions in ER-to-Golgi protein trafficking potentially contribute to ER stress during lipoapoptosis. In this case ER stress would be triggered by protein overload, rather than a disruption of the protein-folding capacity of the ER.

Keywords Apoptosis · Endoplasmic reticulum stress · Lipotoxicity · Pancreatic beta cell · Protein trafficking · Type 2 diabetes · Vesicular stomatitis virus G protein

Electronic supplementary material The online version of this article (doi:10.1007/s00125-009-1506-5) contains supplementary material, which is available to authorised users.

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Abbreviations

BFA	Brefeldin A
ER	Endoplasmic reticulum
FA	Fatty acid
GFP	Green fluorescent protein
VSVG	Vesicular stomatitis virus G protein

Introduction

Apoptosis contributes to the beta cell failure that is essential for the development of type 2 diabetes, and can be recapitulated by chronic exposure of beta cells to saturated fatty acids (FAs). Recent work has implicated endoplasmic

reticulum (ER) stress as a mechanism underlying both lipooptosis in vitro [1, 2] and the loss of beta cell mass observed in human type 2 diabetes and animal models of the disease [2]. ER stress results when the load of client proteins entering the ER exceeds their capacity to be correctly folded. Cells can re-adjust the balance between protein production and folding by activating the unfolded-protein response, but if this fails to resolve the ER stress, apoptosis ensues [1].

Although the enhanced secretory demand that accompanies insulin resistance most probably contributes, ER stress is also triggered by direct actions of saturated FAs on the beta cell. The molecular mechanisms underlying these direct effects are currently unclear. One hypothesis invokes depletion of Ca^{2+} from the ER lumen, which thereby compromises the function of protein-folding chaperones. But Ca^{2+} depletion correlates rather poorly with the capacity of FAs to promote ER stress [3–5]. Here our aim was to investigate an alternative mechanism whereby FAs might induce ER stress by protein overload, secondary to reductions in ER-to-Golgi protein trafficking.

Methods

Cell culture and standard treatments Cells of the mouse insulinoma cell line MIN6 were routinely cultured as previously described [6]. After seeding (24 h) chronic treatments were commenced using Dulbecco's modified Eagle's medium containing 6 mmol/l glucose containing either BSA, or BSA coupled to FA (final 0.4 mmol/l FA:0.92% [wt/vol.] BSA) [6]. Apoptosis ELISA assays and immunoblotting of ER stress markers were performed as detailed previously [2].

Trafficking and folding assays MIN6 cells (3×10^6 per cuvette) were transfected (70% efficiency) with 3 μg DNA encoding the mutated vesicular stomatitis virus G protein VSV-G_{T045} (VSVG) tagged with green fluorescent protein (GFP), or GFP control, by nucleofection (AMAXA Biosystems, Cologne, Germany). Cells were seeded onto coverslips and cultured overnight prior to chronic treatment as above. After 36 h, cells were switched to 40°C for 12 h. Trafficking assays were initiated by transfer to the

Fig. 1 Effects of FAs and ER stress inducers on trafficking of the VSVG protein in MIN6 cells. Cells were nucleofected with VSVG construct and then maintained in tissue culture either under control conditions or in the presence of palmitate (Palm) or oleate (0.4 mmol/l:0.92% BSA for 48 h) or with thapsigargin (Thaps; 300 nmol/l) or tunicamycin (Tunic; 6 $\mu\text{mol/l}$) for 24 h. For the last 12 h the temperature was switched to 40°C, and cyclohexamide (20 $\mu\text{mol/l}$) added for the final 15 min. Cells were then shifted to 32°C for 20 min and fixed and immunocytochemistry was carried out as described in the legend to Fig. 2. **a** Representative images are shown from a single experiment of three. **b** Data from 50 cells in each of three experiments independently scored by two observers are shown. Each image was assigned a score according to the scale: 1, exclusively ER; 2, predominately ER; 3, equivalent ER and Golgi; 4, predominately Golgi; 5, exclusively Golgi. Results represent the mean \pm SEM assigned to each treatment. * $p < 0.05$ vs control or as indicated (Mann–Whitney test)

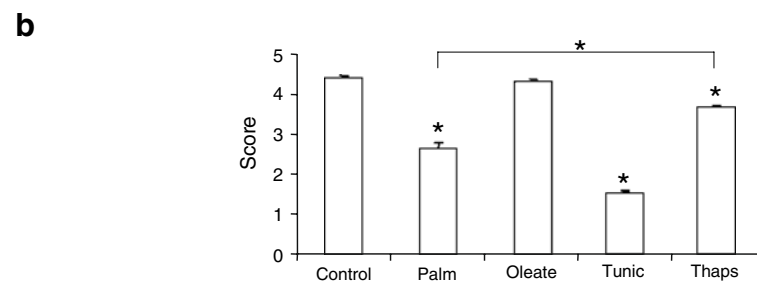
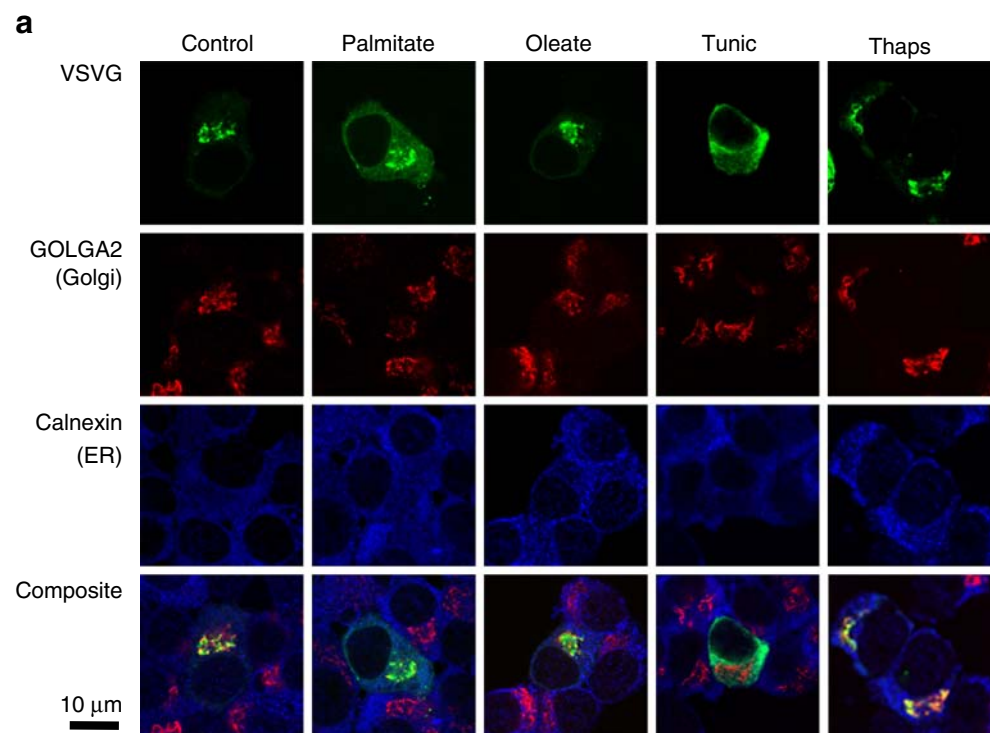
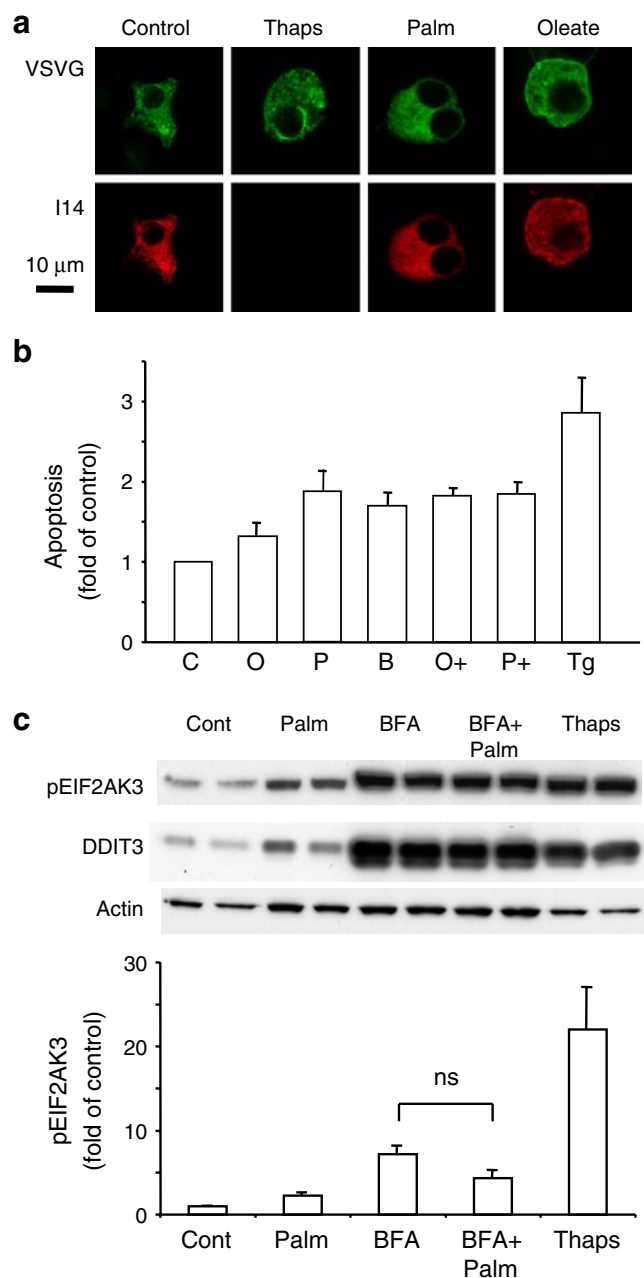


Fig. 2 Effects of FAs and ER stress inducers on VSVG folding (a), apoptosis (b) and ER stress (c) in MIN6 cells. **a** Cells were nucleofected with VSVG construct and then maintained on coverslips in tissue culture either under control conditions or in the presence of palmitate (Palm) or oleate (0.4 mmol/l:0.92% BSA for 48 h) or with thapsigargin (Thaps; 300 nmol/l) for 24 h. For the last 6 h, BFA (1 μ g/ml) was added and the temperature switched to 32°C. Cells were fixed, permeabilised and stained with I14 antibody followed by an anti-mouse secondary antibody conjugated to Cy3. VSVG was visualised by laser scanning confocal microscopy after excitation from a 488 nm laser, and filtration (long pass 515 nm) of the emission. Bound I14 was visualised by excitation at 568 nm and visualised at 590 nm. Representative images from a single experiment of a total of three are shown. **b** Apoptosis was analysed using a cell death detection ELISA, and normalised to the control level in each experiments. Means \pm SEM from four experiments are shown. Apoptosis to all experimental additions except oleate were significantly elevated (ANOVA with Bonferroni correction) relative to control ($p < 0.05$). C, control; P, palmitate; O, oleate; B, BFA; +, FA plus BFA; Tg, thapsigargin; Tn, tunicamycin. **c** Protein extracts were subjected to western blotting for ER stress markers or loading control as indicated (pEIF2AK3, phosphorylated EIF2AK3). Results are shown as representative images from three individual experiments or densitometric analysis of four individual experiments (means \pm SEM). All experimental additions were significantly elevated (ANOVA with Bonferroni correction) relative to control ($p < 0.05$); ns, not significant

permissive temperature of 32°C and addition of 20 μ mol/l cyclohexamide for 0–30 min [7]. After fixation, slides were exposed to primary antibodies for Golgi marker GOLGA2 (also known as GM130) (BD Transduction Labs, Franklin Lakes, NJ, USA) or calnexin (Stressgen, Victoria, BC, Canada). Slides were examined using an SP2 ABOS laser scanning confocal microscope (Leica Microscopes, Wetzlar, Germany) with appropriate excitation wavelengths for individual fluorochromes, and channels scanned in series. Cells moderately overexpressing VSVG were scored for co-localisation with either GOLGA2 or calnexin by two independent observers, with inter-observer variation $< 5\%$. For folding assays, 1 μ g/ml brefeldin A (BFA) was added as the cells were transferred to 32°C. After 6 h, cells were fixed and exposed to the I14 antibody for detection of native VSVG [8].

Results

ER stress is potentially modulated by the rate of ER-to-Golgi trafficking but this segment of the secretory pathway is technically very challenging to assess using endogenous cargo protein. Therefore a reporter protein, the temperature-sensitive VSVG mutant, has been developed as the method of choice for this analysis, at least in readily transfectable cells where the relatively poor expression of the large transgene is negated. This protein is retained in the ER at 40°C but readily exits via the secretory pathway after switching to the permissive temperature of 32°C [7].



Provided its ongoing synthesis is blocked, net transfer from ER to Golgi of the GFP-tagged VSVG can therefore be visualised. We therefore applied the VSVG technique to MIN6 cells that we, and others, have extensively characterised as undergoing a mild ER stress and lipoapoptosis [2, 6, 9] resembling that of primary mouse and human beta cells [4, 9]. As shown in Electronic supplementary material (ESM) Fig. 1, VSVG remains distributed with the ER marker calnexin immediately following the switch to the permissive temperature; after 10 min some co-localises with the Golgi marker GOLGA2, whereas transfer to the Golgi

is virtually complete by 20 min (ESM Fig. 1). Hence, the 20 min time point was employed in subsequent analyses.

Compared with control cells, MIN6 cells pretreated with palmitate retained considerable VSVG in the ER, although there clearly was some co-staining with the Golgi marker (Fig. 1a). This is shown in representative images (Fig. 1a) and in two graphical formats from scoring of multiple cells (ESM Fig. 2; Fig. 1b). The results demonstrate that palmitate delays protein trafficking. This was not apparent in cells pretreated with oleate. Tunicamycin, which promotes ER stress by inhibiting protein glycosylation, markedly inhibited the trafficking of VSVG. This was expected, since VSVG must be glycosylated to enter the secretory pathway. Another inducer of ER stress, thapsigargin, which compromises protein folding by depletion of ER luminal Ca^{2+} , also delayed protein trafficking, but to a significantly lesser extent than palmitate (Fig. 1b).

Another advantage of the VSVG technique is that it facilitates direct assessment of the protein-folding environment of the ER under various experimental conditions. This is achieved by comparing total VSVG levels (estimated via the associated GFP tag) with the signal obtained using the I14 antibody, which detects only the native, or correctly folded, conformation of VSVG [8, 10]. BFA is also employed in these studies to block functional ER-to-Golgi trafficking and thereby localise VSVG for optimal visualisation [10]. Correctly folded VSVG, in the form of strong I14 fluorescence, was detected in the presence of BFA under control conditions, and this was unaltered by pretreatment with palmitate or oleate (Fig. 2a). Conversely, and as expected, thapsigargin compromised the folding environment of the ER such that the native VSVG signal was greatly reduced. Similar effects were seen using tunicamycin (not shown). This disruption of folding probably explains why thapsigargin delays VSVG entry into the secretory pathway (Fig. 1). Importantly, however, this explanation does not account for the even more pronounced delay in trafficking elicited by palmitate, since this did not prevent VSVG folding. Therefore our results suggest that saturated FAs specifically disrupt the actual trafficking process, at a site distal to the folding of cargo protein.

Finally we compared the effects of FAs, BFA and thapsigargin on apoptosis (Fig. 2b), along with phosphorylation of EIF2AK3 (also known as PERK) and levels of DDIT3 (also known as CHOP) as ER stress markers (Fig. 2c). The results demonstrate that ER stress and apoptosis in beta cells is not only triggered by disruption of protein-folding capacity with thapsigargin, but also by protein overload caused by even brief blockade of ER-to-Golgi trafficking with BFA. This establishes that the trafficking defect observed with palmitate could contribute to ER stress and hence lipoapoptosis.

Discussion

Our results clearly demonstrate that saturated, but not unsaturated, FAs specifically slowed ER-to-Golgi protein trafficking. Moreover, disruption of trafficking at this site with BFA was sufficient to cause ER stress, whereas direct induction of strong ER stress with thapsigargin caused only a modest decrease in protein export out of the ER. This places defective trafficking upstream of ER stress. Collectively our data suggest that protein overload should now be considered as a mechanism contributing to ER stress in beta cells chronically exposed to saturated FAs. By slowing the transit of protein from the ER to Golgi, unfolded protein could accumulate under these conditions, even in the absence of changes in the rate of folding, simply because of the build-up of protein in the ER lumen (ESM Fig. 3).

Although the VSVG assay is the method of choice for assessing, directly and specifically, the trafficking of protein between ER and Golgi, it will be important in future studies to confirm our findings using independent techniques, and in primary beta cells. The latter caveat also holds for the other main mechanism previously advanced to explain how saturated FAs promote ER stress in beta cells, namely inhibition of the rate of protein folding in beta cells [4, 9]. Specifically it has been proposed that depletion of ER Ca^{2+} impacts negatively on protein-folding efficiency [4]. However, the effects of different saturated FAs on luminal Ca^{2+} do not always correlate well with ER stress [3–5]. On the other hand, such studies are difficult to quantify accurately, so we certainly do not exclude this possibility. Indeed, it is probable that multiple mechanisms contribute to the overall induction of ER stress. This is especially likely *in vivo*, where the direct effects of saturated FAs on beta cells may be expected to exacerbate the secretory load that is already imposed in trying to overcome peripheral insulin resistance (ESM Fig. 3).

Palmitate pretreatment is also known to impair proinsulin processing [11]. Although it is tempting to speculate that the trafficking defect described here may contribute to this impairment, specific alterations in post-Golgi processing have also been described [11, 12]. Evaluating the relative contribution of these alternative mechanisms would therefore require a better understanding of how palmitate actually reduces protein trafficking, since this would facilitate investigation of whether rescuing the trafficking defect could improve proinsulin conversion. Amongst several potential effects of palmitate, an impact on ER membrane dynamics is perhaps the most likely explanation for the defective trafficking, although alternative mechanisms may involve alterations in signalling or gene expression.

In conclusion, our findings provide further evidence that saturated FAs act directly on beta cells to induce ER stress. In establishing that protein trafficking is specifically

disrupted under these conditions, we provide the important conceptual advance that protein overload potentially contributes as an underlying mechanism. A better understanding of these processes will facilitate the design of future experiments to quantify the relative contributions of protein overload vs protein misfolding to the induction of ER stress during beta cell failure.

Acknowledgements This work was funded by a Project Grant, and Postgraduate Scholarships, from the National Health and Medical Research Council of Australia. We gratefully acknowledge J. Lippincott-Schwartz (NIH, Bethesda, MD, USA) and D. Lyles (Wake Forest University School of Medicine, Winston-Salem, NC, USA) for the generous gifts of the VSVG reporter construct and I14 antibody, respectively. We also thank C. Achard and J. Stoeckli (Garvan Institute of Medical Research, Sydney, NSW, Australia) for critical review of the manuscript.

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

References

- Eizirik DL, Cardozo AK, Cnop M (2008) The role for endoplasmic reticulum stress in diabetes mellitus. *Endocr Rev* 29:42–61
- Laybutt DR, Preston AM, Akerfeldt MC et al (2007) Endoplasmic reticulum stress contributes to beta cell apoptosis in type 2 diabetes. *Diabetologia* 50:752–763
- 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 β -cell apoptosis. *Endocrinology* 147:3398–3407
- Cunha DA, Hekerman P, Ladriere L et al (2008) Initiation and execution of lipotoxic ER stress in pancreatic β -cells. *J Cell Sci* 121:2308–2318
- Gwiazda KS, Yang TL, Lin Y, Johnson JD (2009) Effects of palmitate on ER and cytosolic Ca^{2+} homeostasis in β -cells. *Am J Physiol Endocrinol Metab* 296:E690–E701
- Busch AK, Gurisik E, Sudlow M et al (2005) Increased fatty acid desaturation and enhanced expression of stearyl coenzyme A desaturase protects pancreatic β -cells from lipoapoptosis. *Diabetes* 54:2917–2924
- Presley JF, Cole NB, Schroer TA, Hirschberg K, Zaal KJ, Lippincott-Schwartz J (1997) ER-to-Golgi transport visualized in living cells. *Nature* 389:81–85
- Lefrancois L, Lyles DS (1982) The interaction of antibody with the major surface glycoprotein of vesicular stomatitis virus. I. Analysis of neutralizing epitopes with monoclonal antibodies. *Virology* 121:157–167
- Lai E, Bikopoulos G, Wheeler MB, Rozakis-Adcock M, Volchuk A (2008) Differential activation of ER stress and apoptosis in response to chronically elevated free fatty acids in pancreatic β -cells. *Am J Physiol Endocrinol Metab* 294:E540–E550
- Nehls S, Snapp EL, Cole NB et al (2000) Dynamics and retention of misfolded proteins in native ER membranes. *Nat Cell Biol* 2:288–295
- Furukawa H, Carroll RJ, Swift HH, Steiner DF (1999) Long-term elevation of free fatty acids leads to delayed processing of proinsulin and prohormone convertases 2 and 3 in the pancreatic β -cell line MIN6. *Diabetes* 48:1395–1401
- Jeffrey KD, Alejandro EU, Luciani DS et al (2008) Carboxypeptidase E mediates palmitate-induced β -cell ER stress and apoptosis. *Proc Natl Acad Sci USA* 105:8452–8457