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Thapsigargin Down-Regulates Protein Levels of GRP78/BiP in INS-1E Cells

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ABSTRACT

Pancreatic β-cells have a well-developed endoplasmic reticulum (ER) and express large amounts of chaperones and protein disulfide isomerases (PDI) to meet the high demand for synthesis of proteins. We have observed an unexpected decrease in chaperone protein level in the β-cell model INS-1E after exposure to the ER stress inducing agent thapsigargin. As these cells are a commonly used model for primary β-cells and has been shown to be vulnerable to ER stress, we hypothesize these cells are incapable of mounting a chaperone defense upon activation of ER stress. To investigate the chaperone expression during an ER stress response, induced by thapsigargin in INS-1E cells, we used quantitative mass spectrometry based proteomics. The results displayed a decrease of GRP78/BiP, PDIA3 and PDIA6. Decrease of GRP78/BiP was verified by Western blot and occurred in parallel with enhanced levels of p-eIF2α and CHOP. In contrast to INS-1E cells, GRP78/BiP was not decreased in MIN6 cell or rat and mouse islets after thapsigargin exposure. Investigation of the decreased protein levels of GRP78/BiP indicates that this is not a consequence of reduced mRNA expression. Rather the reduction results from the combined effect of reduced protein synthesis and enhanced proteosomal degradation and possibly also degradation via autophagy. Induction of ER stress with thapsigargin leads to lower protein levels of GRP78/BiP, PDIA3 and PDIA6 in INS-1E cells which may contribute to the susceptibility of ER stress in this β-cell model. J. Cell. Biochem. 113: 1635–1644, 2012.

KEY WORDS: GRP78/BIP; THAPSIGARGIN; β-CELL; ENDOPLASMIC RETICULUM; PROTEIN DEGRADATION; DIABETES

The two major forms of diabetes, type 1 (T1D) and type 2 (T2D) share, although with different pathogenesis, the concept of a reduction in functional pancreatic β-cell mass [Cnop et al., 2005]. One cellular response induced both in T1D and T2D is the unfolded protein response (UPR) [Kharroubi et al., 2004; Özcan et al., 2004; Cardozo et al., 2005; Laybutt et al., 2007; Ortsäter and Sjöholm, 2007; Eizirik et al., 2008]. Normally, the efforts mounted by the UPR restore the intra-organelle milieu and are therefore cytoprotective. The UPR is initiated by accumulation of misfolded proteins in the ER that activates three distinct pathways, each controlled by a specific protein; eukaryotic translation factor 2-alpha kinase 3 (PERK), endoplasmic reticulum (ER) to nucleus signaling 1 (IRE1) and activating transcription factor 6 (ATF6), respectively [Boyce and Yuan, 2006]. Activation of PERK promotes the phosphorylation and inhibition of the translation initiation factor, eIF2α [Prostko et al., 1993]. The resulting attenuation of general protein synthesis prevents further overloading of the ER folding machinery. Activation of IRE1 [Calfon et al., 2002] and ATF6 [Haze et al., 1999] promotes the expression of ER chaperones and thereby increases the capacity of the ER to fold the accumulated polypeptides. These ER chaperones also function as part of the quality control system that targets misfolded proteins for ER-associated protein degradation [Plemper et al., 1997]. Thus, the UPR acts to alleviate ER stress by inhibiting general protein translation, increasing folding capacity, and promoting degradation of misfolded proteins.

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Experimental manipulation of the UPR system in β-cells generally leads to attenuated function and cell death. For example, perturbation of IRE1 signaling in β-cells through sustained production of spliced X-box binding protein 1 (XBP1) evokes β-cell dysfunction and apoptosis [Allagnat et al., 2010]. Deletion of PERK gene results in progressive destruction of β-cells in both humans and mice [Delepine et al., 2000; Harding et al., 2001]. In humans, mutations in the PERK gene cause Wolcott–Rallison syndrome, which manifests as an infantile-onset, insulin-requiring diabetes [Delepine et al., 2000]. In *perk*$^{-/-}$ mice, there is a progressive loss of β-cells and diabetes develops within the first few weeks after birth [Harding et al., 2001]. Even more severe β-cell dysfunction is seen in mice with a homozygous serine 51 alanine point mutation in their eIF2α gene [Scheuner et al., 2001; Back et al., 2009]. In addition, inhibition of eIF2α dephosphorylation provokes β-cell apoptosis [Cnop et al., 2007; Ladriere et al., 2010].

While IRE1 and PERK pathways can transduce ER stress cytotoxicty, ATF6 signaling through activation of chaperone proteins is primarily considered to provide cytoprotective inputs [Wu et al., 2007; Yamamoto et al., 2007]. We have observed that INS-1E cells when treated with thapsigargin, a SERCA pump inhibitor that depletes calcium from the ER, that has been used in multiple investigations to induce UPR and ER stress in both β-cells [Karaskov et al., 2006; Yusta et al., 2006] and other cell types [Rutkowski et al., 2006], actually had lowered cellular content of the important chaperone glucose-regulated protein 78/immunoglobulin heavy-chain binding protein (GRP78/BiP). Against this background we performed experiments to find out by which mechanism thapsigargin decreased GRP78/BiP in INS-1E cells.

MATERIALS AND METHODS

CULTURE OF CELLS AND PRIMARY ISLETS

The rat insulinoma cell line INS-1E [Merglen et al., 2004] (a kind gift from Professor Claes Wollheim) was used between passages 71–80 and maintained in RPMI 1640 medium (SVA, Uppsala, Sweden) containing 11.1 mM glucose supplemented with 10% FBS (Invitrogen, Carlsbad, CA), 10 mM HEPES (Invitrogen), 2 mM L-glutamine (SVA), 1 mM sodium pyruvate (Sigma–Aldrich, St. Louis, MO), 50 μM β-mercaptoethanol (Sigma–Aldrich), and antibiotics (6 mg/ml pencillin G and 5 mg/ml streptomycin sulfate [Invitrogen]) at 37°C in a humidified atmosphere containing 5% CO_2. The mouse insulinoma cell line MIN6 [Miyazaki et al., 1990] (a kind gift from Dr. Jun-ichi Miyazaki) was used between passages 25–30 and maintained in DMEM medium (Invitrogen) containing 25 mM glucose supplemented with 15% FBS, 2 mM L-glutamine, 50 μM β-mercaptoethanol and antibiotics (6 mg/ml pencillin G and 5 mg/ml streptomycin sulfate) at 37°C in a humidified atmosphere containing 5% CO_2.

Islets of Langerhans were isolated from male Wistar rats and C57Bl/6J mice (Scanbur, Sweden) and maintained in culture as previously described [Sargsyan et al., 2008]. Animal handling was performed according to national law and approved by local ethical committee.

DIGESTION AND iTRAQ LABELING

INS-1E cells for iTRAQ analysis were plated in six-well plates at a density of 450,000 cells per well in 3 ml medium for 72 h prior to treatment. Cells were exposed to 200 nM thapsigargin (Sigma–Aldrich) for 0, 4, 16, and 24 h. After exposure, cells were washed with PBS and lyzed in a buffer containing 150 mM NaCl, 20 mM Tris, 0.1% SDS, 1% Triton X-100, 0.25% Na-deoxycholate, 1 mM Na_3VO_4, 50 mM NaF, 2 mM EDTA, and Protease inhibitory cocktail (Sigma–Aldrich) on ice for 30 min. Protein concentration was determined with Bio-Rad DC protein assay (Bio-Rad Laboratories, Hercules, CA) and equal amounts of protein (50 μg) were acetone-precipitated. The pellets were dissolved in dissolution buffer containing triethylammonium bicarbonate from the iTRAQ 8plex kit (Applied Biosystems, Foster City, CA). Denaturant containing SDS from the iTRAQ kit was added, proteins were reduced by adding tris-(2-carboxyethyl) phosphine and alkylated by methyl methanethiosulfonate (MMTS) from the iTRAQ kit. Trypsin (TPCK treated, Applied Biosystems) was added and the samples were incubated overnight at 37°C. The iTRAQ labeling reagents 113, 114, 115, 116, 117, 118, 119, and 121 were done according to the manufacturer's instructions. The peptides were analyzed with MALDI-TOF/TOF. Peptide identification from the MALDI-TOF/TOF data was carried out using the Paragon algorithm [Shilov et al., 2007] in the ProteinPilot 2.0 software package (Applied Biosystems). Searches were performed against the IPI database (build 3.60) limited to rat sequences. For details according the digestion and iTRAQ labeling, Nano-LC-Maldi MS/MS analysis and peptide and protein identification, see supplementary material.

PROTEIN EXTRACTION, SDS–PAGE AND WESTERN BLOT ANALYSIS

INS-1E cells and MIN6 cells for Western blot analysis were plated in six-well plates at a density of 450,000 cells per well in 3 ml medium for 72 h before treatment. For evaluation of the protein levels of GRP78/BiP, and confirmation of induction of ER stress, cells were exposed to 200 nM thapsigargin for 0, 4, 16 and 24 h or exposed to different concentrations of thapsigargin for 16 h. For inhibition of protein synthesis, cells were exposed to 10 μM cycloheximide (Sigma–Aldrich) for 24 h. For experiments involving proteosome inhibition, INS-1E cells were exposed to a combination of 200 nM thapsigargin and 5 μM of the proteosome inhibitor lactacystin (Calbiochem, San Diego, CA) for 24 h. For experiments involving inhibition of autophagy, INS-1E cells were exposed to 5 mM 3-methyladenine (3-MA (Sigma–Aldrich)) in combination with 200 nM thapsigargin. All chemicals were prepared immediately prior to usage to minimize activity loss. In all experiments untreated cells received equal amount of solvent (DMSO) as treated cells.

After exposure to the above agents, INS-1E and MIN6 cells were washed in PBS and lyzed in buffer as described above. Equal amounts of protein were then separated by 10–15% SDS–PAGE and transferred to Immun Blot™ PVDF membranes (Bio-Rad Laboratories). Immunoblot analysis was performed using primary antibodies against GRP78/BiP (Abcam, Cambridge, UK, Cat# ab21685), p-eIF2α (Cat# 9721), eIF2α (Cat# 9722), p-JNK (Cat# 9251), JNK (Cat# 9252), LC3B (Cat# 2775), cleaved caspase 3 (Cat# 9661) (Cell Signaling Technology, Danvers, MA), CHOP (Cat# sc-575), Chromogranin-A (Cat# sc-13090), cleaved spectrin α II (Cat# sc-23464), and p53 (Cat#

sc-6243) (Santa Cruz Biotechnology, Santa Cruz, CA). Horesradish peroxidase-conjugated goat anti-rabbit IgG was used as a secondary antibody (Santa Cruz Biotechnology). The protein-specific signals were detected using Enhance Chemiluminescence (GE-healthcare, Fairfield, CT). The signals were quantified using Molecular Imager ChemiDoc XRS with Quantity One Software v. 4.6.5 (Bio-Rad Laboratories). Coomassie staining (0.1% Brilliant Blue R-250 in 40% methanol and 10% acetic acid) was used for protein normalization (BioRad Laboratories).

Rat and mouse islet drug exposure started 24 h after isolation with 200 nM thapsigargin for 24 h. Control islets were exposed to DMSO alone. After exposure, islets were washed with ice-cold PBS and lyzed in the same buffer used for the cell lines. The lysate was then sonicated and separated by SDS–PAGE similarly as the lysate from cell lines.

PROTEIN SYNTHESIS

To monitor protein synthesis, INS-1E cells were exposed to 200 nM thapsigargin for 4 h. Cells were then starved in a medium lacking L-methionine and L-cysteine for 1 h. Next, Met-[^{35}S]-label IS-103 isotope, (Biotech-IgG, Denmark) was added and cells were left for one additional hour. After labeling, cells were harvested, lyzed and radioactivity was measured in equal protein loads in a β-counter (Perkin-Elmer).

RNA EXTRACTION, cDNA SYNTHESIS, AND QUANTITATIVE RT-PCR

INS-1E cells for mRNA analysis were plated in 12-well plates at a density of 300,000 cells per well in 2 ml medium for 72 h before treatment. The cells were exposed to 200 nM thapsigargin for 0, 4, 16, and 24 h. Total RNA was isolated using Aurum™ Total RNA Mini kit (BioRad Laboratories). Total RNA was reverse transcribed using iScript™ cDNA Synthesis kit (BioRad Laboratories). The resulting cDNA was used for real-time PCR analysis using Maxima™ SYBR Green/Fluorescein qPCR Master Mix (Fermentas, Burlington, ON, Canada). Normalization was performed using the housekeeping gene *Rattus norvegicus* ribosomal protein L13A (Rpl13a), whose expression is not modified by thapsigargin. For quantification, the following formula was used: target amount $= 2 - \Delta\Delta Ct$, where $\Delta\Delta Ct = \{[Ct \text{ (target gene sample)} - Ct \text{ (Rpl13a sample)}] - [Ct \text{ (control sample)} - Ct \text{ (Rpl13a control)}]\}$ [Livak and Schmittgen, 2001]. For the primer sequences used in this study, see supplementary material.

MEASUREMENT OF XBP1 mRNA SPLICING

Total RNA was isolated and cDNA synthesized from INS-1E cells as described above after exposure to 200 nM thapsigargin for 0, 4, 16, and 24 h. Rat XBP1 cDNA was amplified using iProof Master Mix (Fermentas) and XBP1 primers, flanking the 26 bp splicing site [Calfon et al., 2002], which contains a *Pst*I restriction site (CTGCAG). For primer sequences, see supplementary material. The PCR product was incubated with *Pst*I (Fermentas) for 30 min at 37°C and the digested product was then separated on a 2% agarose gel and visualized using ethidium bromide. The gel shows one band at 746 bp for spliced XBP1 and two bands at 458 and 314 bp for the nonspliced XBP1.

APOPTOSIS ASSAY

The cell death detection kit ELISA^{PLUS} (Roche Diagnostics) was used to monitor thapsigargin-induced apoptosis. INS-1E cells were plated in 24-well plates at a density of 200,000 cells per well in 1 ml medium for 72 h before treatment. The cells were exposed to 200 nM thapsigargin for 24 h. After exposure, the cells were washed in PBS twice, lyzed, and oligonucleosomes were quantified according to the manufacturer's instructions. The absorbance was measured at 405 nm using Magellan software v 6.55 (TECAN Group Ltd., Männedorf, Switzerland).

STATISTICAL ANALYSIS

Data are presented as mean ± SEM. Comparisons were performed using Student's *t*-test or one-way ANOVA with Dunnett post-hoc test using GraphPad Prism 5. A P-value < 0.05 was deemed as statistically significant.

RESULTS

THAPSIGARGIN TIME- AND DOSE-DEPENDENTLY DECREASE GRP78/BiP PROTEIN LEVELS IN INS-1E CELLS

Treatment with thapsigargin leads to emptying of ER Ca^{2+} stores and induces a profound ER stress response along with induction of apoptosis [Luciani et al., 2009]. INS-1E cells were treated with 200 nM thapsigargin for 0, 4, 16, and 24 h, after which cellular proteins were isolated and subjected to iTRAQ labeling using eight different isobaric tags. Two independent samples from each time point were combined in the analysis. From the positive identification of 269 unique proteins, 8 were classified as chaperones, 2 as protein disulfide isomerases (PDIs), and 2 as peptidylprolyl isomerases (PPI). Of the chaperones, four are cytoplasmic, three mitochondrial and one localized to the ER. Both heat shock protein 90β (cytoplasmic) and GRP78/BiP (ER) decreased with time, ending at 68 ± 0.6% and 38 ± 0% of their baseline levels, respectively (Fig. 1A). Other chaperones remained unaffected. The PDIs, PDIA3, and PDIA6 (both localize to the ER), decreased with time, ending at 71 ± 0% and 29 ± 0%, respectively, compared to the control (Fig. 1A). Of the PPIs identified, PPIA (cytoplasmic) and PPIB (ER) levels were not affected by thapsigargin treatment (not shown). From the very same samples we investigated protein expression of GRP78/BiP with Western blot. The analysis showed significantly decreased protein levels of GRP78/BiP at the time points 16 and 24 h with a 31 ± 7% and 29 ± 7% reduction, respectively, compared to the control (Fig. 1B,C), thus confirming our iTRAQ findings. Reduction of GRP78/BiP occurred in parallel with enhanced phosphorylation status of eIF2α (p-eIF2α), decreased protein synthesis and elevated protein levels of CHOP (Fig. 1C,D). The exposure of cells to different concentrations (0–300 nM) of thapsigargin for 16 h also resulted in decreased protein levels of GRP78/BiP with the maximal effect at 200 nM (Fig. 1E). Furthermore, in our system, 200 nM of thapsigargin induced IRE1 pathway activity as evidenced by enhanced alternative splicing of XBP1 and increased phosphorylation of JNK (Fig. 1F,G). As expected, elevated levels of cleaved caspase-3 and increased DNA fragmentation was also elicited by thapsigargin (Fig. 1H,I). We could not detect any GRP78/BiP protein released to cell culture medium during

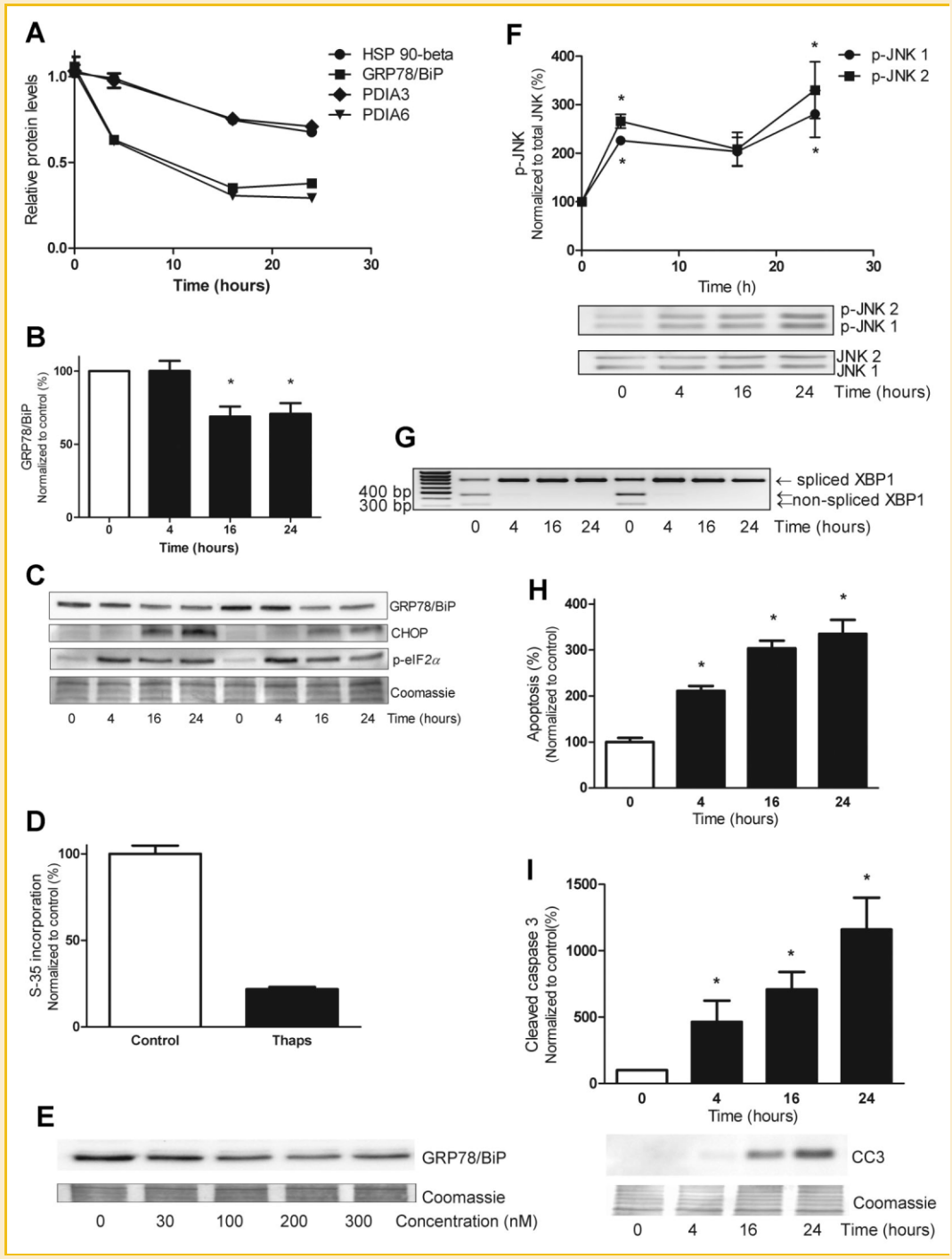

Fig. 1. Thapsigargin induces ER stress and down-regulates protein levels of chaperones and PDIs. INS-1E cells were exposed to 200 nM thapsigargin for 0, 4, 16, and 24 h or for indicated concentrations for 16 h. A: Cellular protein were isolated and subjected to iTRAQ labeling using eight different isobaric tags. Protein identification and quantification was performed with LC/MS/MS. Data is presented as mean ± SEM of two replicates. Data is normalized to time 0. B: Protein levels of GRP78/BiP. Data were normalized to the control condition (0 h that includes DMSO). Bars represent mean ± SEM, n = 5. $^{*}P < 0.05$ for a chance difference versus time 0. C: Blots showing protein levels of GRP78/BiP. Confirmation of induction of ER stress with blots showing CHOP and phosphorylation status of eIF2α after the same treatment. The time courses represent replicates from two independent experiments. Fifteen micrograms of protein was loaded per lane. D: Protein synthesis in cells exposed to 200 nM thapsigargin for 4 h after Met-[^{35}S]-label IS-103 isotope incorporation, n = 4. E: Blots showing protein levels of GRP78/BiP after treatment with different concentrations of thapsigargin for 16 h. Twenty micrograms of protein was loaded per lane. F: Western blot showing phosphorylation status of JNK 1 and 2. Data is normalized to total JNK. Data is presented as mean ± SEM, n = 3. $^{*}P < 0.05$ for a chance difference versus time 0. Thirty micrograms of protein was loaded per lane. G: The PCR product with size 746 bp corresponds to the spliced form of XBP1. The band at 458 and 314 bp corresponds to the nonspliced form of XBP1. H: Apoptosis was evaluated by using a cell death detection ELISA kit measuring the levels of cytoplasmic oligonucleosomes. The data were normalized to the control condition (0 h). Bars represent mean ± SEM, n = 4. $^{*}P < 0.05$ for a chance difference versus time 0. I: Analysis of protein levels of cleaved caspase 3 by Western blot, n = 3. Thirty micrograms of protein was loaded per lane.

thapsigargin exposure for up to 24 h as has been reported to occur from human rabdomiosarcoma cells [Delpino and Castelli, 2002]. On the other hand we could readily detect Chromogranin-A release (data not shown). Taken together, these data shows that thapsigargin do induce many of the expected effects in INS1-E cells but do not increase the expression of chaperones. In opposite, the expression of GRP78/BiP is decreased upon thapsigargin treatment.

THAPSIGARGIN-INDUCED DECREASE IN THE PROTEIN LEVELS OF GRP78/BiP DOES NOT OCCUR IN MIN6 CELLS, ISOLATED MOUSE ISLETS, OR RAT ISLETS

In order to investigate the protein levels of GRP78/BiP in different cell types than INS-1E cells, MIN6 cells, mouse islets and rat islets were treated with 200 nM thapsigargin for 24 h. Analysis of the protein levels of GRP78/BiP with Western blotting showed decreased levels in INS-1E cells, while the treatment did not decrease the protein levels of GRP78/BiP in MIN6 cells, mouse islets or rat islets (Fig. 2A,B). The phosphorylation status of p-eIF2α and protein levels CHOP was analyzed to confirm induction of ER stress after the treatment (Fig. 2A).

THAPSIGARGIN INCREASES GRP78/BiP AND CHOP mRNA LEVELS IN INS-1E CELLS

To investigate whether the decreased protein levels of GRP78/BiP and PDIA3 (observed in Fig. 1) are secondary to decreased expression of their mRNAs, INS-1E cells were treated with 200 nM thapsigargin for 0, 4, 16, and 24 h, total RNA was isolated and cDNA synthesized. The mRNA levels of the ER stress marker CHOP were also measured. The mRNA levels of GRP78/BiP were increased with time, ending at 271 ± 34% compared to the control (Fig. 3A), the mRNA levels of PDIA3 were not changed with time compared to the control (Fig. 3B), as expected, the mRNA levels of CHOP were also increased with time, ending at 1018 ± 247% compared to the baseline level (Fig. 3C). Our conclusion from these data is that thapsigargin activates the ATF6 signaling pathway but increased GRP78/BiP mRNA is not translated into increased protein levels.

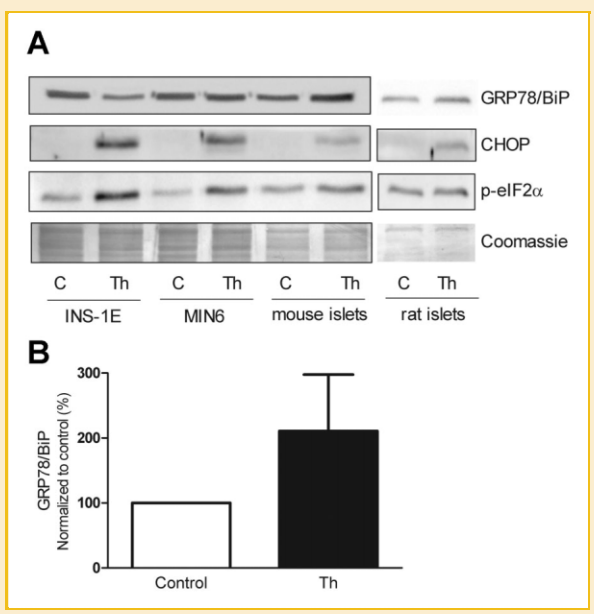

Fig. 2. Investigation of GRP78/BiP protein levels in different cells. INS-1E cells, MIN6 cells, isolated mouse islets and isolated rat islets were cultured in the absence (C) or presence of 200 nM thapsigargin (Th) for 24 h. A: The protein levels of GRP78/BiP, CHOP and phosphorylation status of eIF2α (p-eIF2α) and were analyzed by Western blot. Fifteen micrograms of protein was loaded per lane. B: Protein levels of GRP78/BiP in isolated rat islets, n = 4.

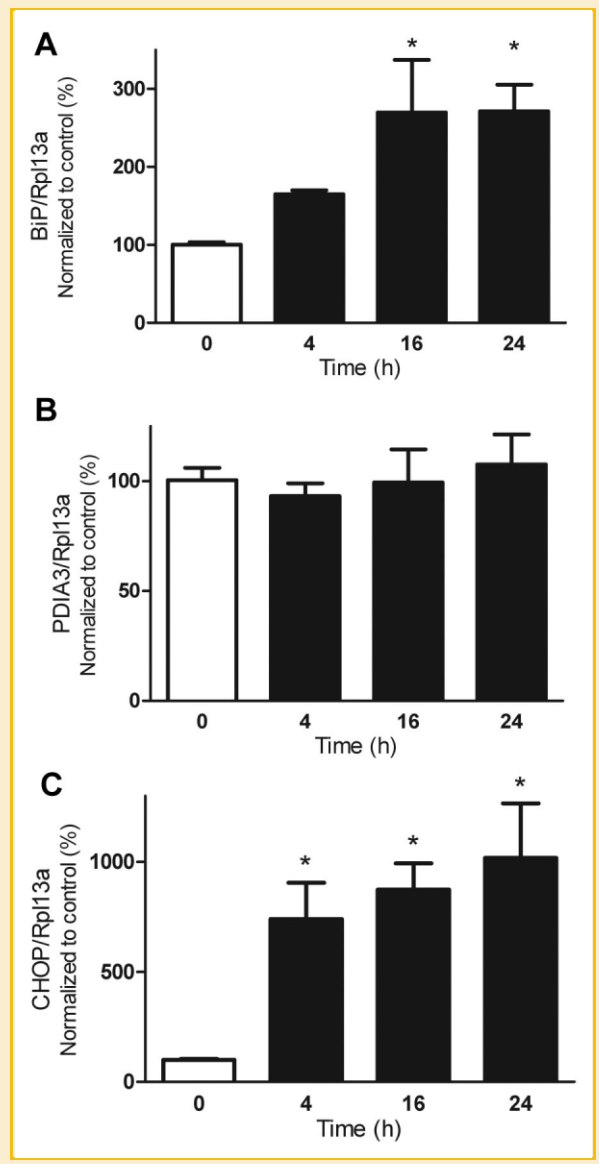

Fig. 3. Differential effects of thapsigargin on mRNA levels of (A) GRP78/BiP, (B) PDIA3, and (C) CHOP. INS-1E cells were exposed to 200 nM thapsigargin for 0, 4, 16, and 24 h. mRNA levels were quantified by qPCR and data was normalized to Rpl13A. Bars represent means ± SEM, n = 4. $^{*}P < 0.05$ for a chance difference versus time 0.

INHIBITION OF PROTEIN SYNTHESIS DOES NOT FULLY EXPLAIN THE DECREASED PROTEIN LEVELS OF GRP78/BiP BY THAPSIGARGIN

Thapsigargin treatment induced phosphorylation of eIF2α (Fig. 1C) and decreased protein synthesis (Fig. 1D). If the observed reduction in GRP78/BiP protein levels would be the mere consequence of a reduction in protein synthesis any general inhibitor of protein synthesis would cause a similar reduction in GRP78/BiP. To test this hypothesis INS-1E cells were exposed to 200 nM thapsigargin or 10 μM cycloheximide for 24 h. At these concentrations, both cycloheximide and thapsigargin caused a similar inhibition of protein synthesis (data not shown). However, the GRP78/BiP levels were only slightly decreased after treatment with cycloheximide (8.6 ± 3.7% reduction compared to the control), while thapsigargin decreased GRP78/BiP levels by 41 ± 1.9% compared to the control (Fig. 4). To ascertain the bioactivity of cycloheximide, we investigated the protein levels of p53 that is a protein with a short half-life and therefore responds rapidly. Exposure of INS-1E to 10 μM cycloheximide decreased the protein levels of p53 with 75% during the first hours of treatment, thus confirming the bioactivity of the agent (Fig. S1). This line of data shows that inhibition of protein synthesis does not fully explain the decreased protein levels of GRP78/BiP by thapsigargin. We therefore examined if degradation of GRP78/BiP occurred after thapsigargin treatment.

INHIBITION OF PROTEOSOMAL PROTEIN DEGRADATION AND AUTOPHAGY REVERSES A THAPSIGARGIN-INDUCED REDUCTION IN GRP78/BiP PROTEIN LEVELS IN INS-1E CELLS

There are two principal pathways for cellular degradation of proteins, that is, proteosomal degradation and autophagy [Ding and Yin, 2007]. In order to address the involvement of the proteosome on the decreased protein levels of GRP78/BiP, we employed the proteasome inhibitor lactacystin. To confirm the bioactivity of lactacystin, we investigated the protein levels of p53 after exposure of INS-1E cells to 5 μM lactacystin for 0, 1, 2, 3, 4, and 5 h. Lactacystin increased protein levels of p53 over time, thus showing that the inhibitor is bioactive (Fig. S2). INS-1E cells were exposed to 200 nM thapsigargin, 5 μM lactacystin or a combination thereof for 24 h. Western blot analysis showed decreased protein levels of GRP78/BiP after exposure to thapsigargin with a reduction of 43 ± 3.7%, whereas cells exposed to the combined treatment showed a reduction of only 26 ± 2.1% compared to untreated cells (Fig. 5), indicating a contribution of proteosomal protein degradation. Lactacystin alone did not significantly influence GRP78/BiP protein levels (Fig. 5).

A second cellular pathway for protein disposal is degradation via autophagy [Yorimitsu and Klionsky, 2007]. Exposure of INS-1E cells to 200 nM thapsigargin produced a clear induction of autophagy, as assessed by induction of Apg5 mRNA expression and the conversion of LC3B-I (microtubule-associated protein 1 light chain 3 B) to LC3B-II (Fig. S3). To elucidate the contribution of autophagy to the reduced GRP78/BiP protein levels, we used the autophagy inhibitor 3-MA, which inhibits autophagy by blocking autophagosome formation via the inhibition of type III Phosphatidylinositol 3-kinases. The conversion of LC3B-I to LC3B-II after treatment with 200 nM thapsigargin for 24 h was found to increase with 990 ± 65%, while treatment with thapsigargin in combination with 3-MA produced a 780 ± 56% increase compared to the control (Fig. 6A). Western blot analysis revealed a decrease of GRP78/BiP with 25 ± 4.2% after treatment with 200 nM thapsigargin for 24 h, while the combined treatment with 200 nM thapsigargin and 5 mM 3-MA produced a decrease with 9.4 ± 5.9% compared to the control. The levels of GRP78/BiP after treatment with 3-MA alone increased with 24 ± 11% (Fig. 6B). This indicates that degradation via autophagy can explain a small part of the decreased protein levels of GRP78/BiP.

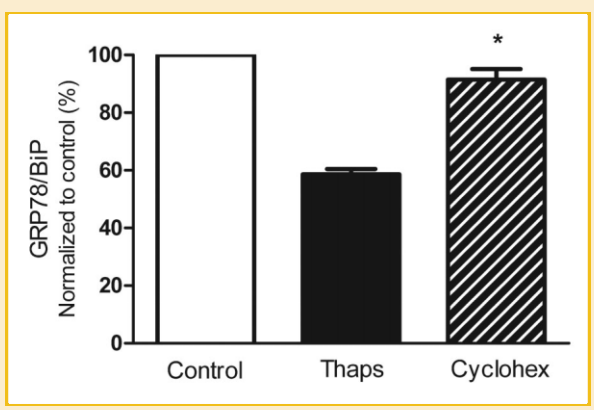

Fig. 4. Effects of inhibition of protein synthesis on GRP78/BiP protein levels. INS-1E cells were exposed to 200 nM thapsigargin (Thaps) or 10 μM cycloheximide (Cyclohex) for 24 h. Cells were lyzed and protein levels of GRP78/BiP were analyzed with Western blot. Data were normalized to the control condition (0 hours). Bars represent means ± SEM, n = 13. $^{*}P < 0.05$ for a chance difference between Cyclohex and control.

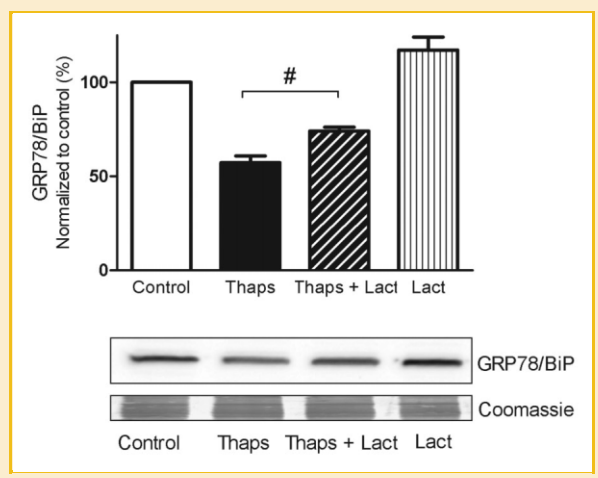

Fig. 5. Effects of inhibition of proteosomal degradation on thapsigargin-induced changes in GRP78/BiP protein levels. A: INS-1E cells were exposed to 200 nM thapsigargin (Thaps), a combination of 200 nM thapsigargin, and 5 μM lactacystin (Lac) or lactacystin alone for 24 h. Protein levels of GRP78/BiP were analyzed with Western blot. Fifteen micrograms of protein was loaded per lane. Data were normalized to the control condition (0 h), Thaps, Thaps + Lact, n = 8; Lact, n = 5. $^{\#}P < 0.05$ for a chance difference between Thaps and Thaps + Lact.

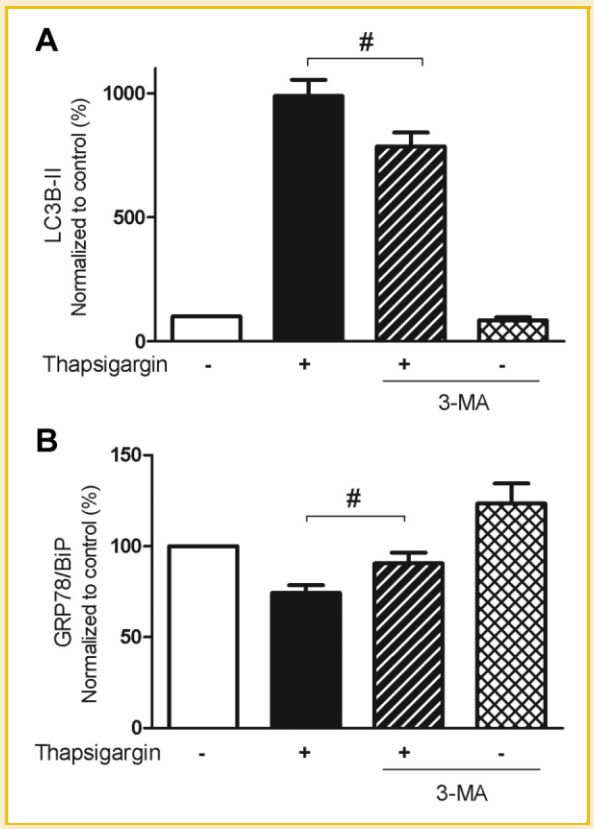

Fig. 6. Effects of inhibition of autophagy with 3-MA on thapsigargin effects on GRP78/BiP protein levels. INS-1E cells were exposed to 200 nM thapsigargin, 5 mM 3-MA or a combination of 200 nM thapsigargin and 5 mM 3-MA for 24 h. Conversion of LC3B-I to LC3B-II (A) and protein levels of GRP78/BiP (B) were analyzed by Western blot, n = 6. $^{\#}P < 0.05$ for a chance difference between thapsigargin and thapsigargin + 3-MA.

DISCUSSION

This study shows a dysfunctional regulation of the levels of ER chaperones GRP78/BiP, PDIA3, and PDIA6 during thapsigargin treatment of INS-1E cells. The INS-1E cell line is commonly used in diabetes research and has been shown to have a stable differentiated β-cell phenotype and exhibit secretory response to glucose at physiological concentrations [Merglen et al., 2004]. The cell line has been used in numerous studies addressing mechanisms of ER stress in pancreatic β-cells [Cardozo et al., 2005; Cunha et al., 2008; Sargsyan et al., 2008].

Thapsigargin is an inhibitor of the SERCA pump that induces ER stress by disrupting ER Ca^{2+} homeostasis. Although a chemical inducer, the data obtained by using this compound can have some implication for the understanding of the pathogenesis of both type 1 and type 2 diabetes. Treatment of pancreatic β-cell with both cytokines [Cardozo et al., 2005] and fatty acids [Cunha et al., 2008; Gwiazda et al., 2009] leads to a lowering of ER Ca^{2+} levels. At least in the former case this is a consequence of reduced SERCA expression. In addition, the SERCA 3 isoform was found to be decreased in the Goto-Kakizaki rat model of type 2 diabetes [Varadi et al., 1996].

In mammalian cells, ER chaperone expression is controlled by ATF6α transcription factor [Haze et al., 1999; Yamamoto et al., 2007]. The expected result after treating INS-1E cells with thapsigargin would be an increase in GRP78/BiP and PDI protein levels. In contrast, we have observed the direct opposite effect, a paradoxical decrease. These data are in agreement with those obtained when using cyclopiazonic acid (CPA), another SERCA pump inhibitor [D'Hertog et al., 2010]. CPA caused a similar degree of apoptosis as observed in this study with thapsigargin and a 2D-DIGE analysis of the proteome demonstrated that GRP78/BiP decreased after CPA exposure. Hence, these two proteomic approaches, 2D-DIGE in the CPA study [D'Hertog et al., 2010] and iTRAQ in the present study gave the same result for GRP78/BiP. A difference between the two studies was that we could confirm our iTRAQ data by Western blotting. This indicates that the decreased levels of GRP78/BiP that we see are not due to post-translational modifications (for example glycosylation), as suggested as an explanation for the discrepancy between results obtained by 2D-DIGE and Western blotting in the CPA study [D'Hertog et al., 2010]. As neither iTRAQ nor Western blotting, with the antibodies used here, are able to discriminate between different forms of GRP78/BiP, the observed decrease must be attributed to a decrease in the total levels of GRP78/BiP.

As induction of chaperone expression occurs to maintain ER function during conditions of ER stress, the data presented here provides one explanation as to why the INS-1E cells are vulnerable to conditions of ER stress. Investigation of the GRP78/BiP protein levels in MIN6 cells and isolated islets from rat and mouse do not show the same effect compared to the situation in INS-1E cells. Comparison of the ER stress markers CHOP and p-eIF2α in the different cell types also shows that the induction of ER stress is less pronounced in MIN6 cells and the islets compared to INS-1E cells. The fact that MIN6 cells and islets do not display the same type of decreased protein levels of GRP78/BiP, compared to INS-1E cells after treatment with thapsigargin may explain why INS-1E cells are more vulnerable than MIN6 cells and islets to conditions of ER stress [Lai et al., 2008; Sargsyan et al., 2008]. Indeed, over expression of GRP78/BiP in these cells reduced susceptibility to thapsigargin-induced apoptosis [Lai et al., 2008]. Since INS-1E cells is commonly used in β-cell research we wanted to further investigate what happens to the GRP78/BiP protein in INS-1E cells after treatment with thapsigargin.

Activation of ATF6α signaling can be monitored by measuring the appearance of p50ATF6α [Yoshida et al., 2001; Sargsyan et al., 2004]. However, attempts to detect p50ATF6α after induction of ER stress by palmitate in INS-1E cells were unsuccessful [Sargsyan et al., 2008]. Despite this, it is reasonable to believe that ATF6 signaling is activated in our system. Indeed, GRP78/BiP mRNA levels accumulate over time during thapsigargin treatment. This observation also tells that decreased mRNA expression cannot explain the reduction in GRP78/BiP or PDIA3 protein, since their mRNA levels are not decreased during the thapsigargin time course.

Decreased protein synthesis can provide an alternative explanation. Indeed, thapsigargin induces prompt phosphorylation of eIF2α

followed by inhibition of protein synthesis [Prostko et al., 1993], by 75% in this study. A similar degree of protein synthesis inhibition was obtained by treating INS-1E cells with cycloheximide. Treatment of INS-1E cells with cycloheximide for 24 h had only minor effects on GRP78/BiP levels. Hence, inhibition of protein synthesis per se can explain some, but not all, of the thapsigargin effect on GRP78/BiP protein levels. Therefore, we went on to investigate whether protein degradation by the proteosome could explain the thapsigargin effect. Induction of ER stress, mainly via the IRE1 signaling pathway, enhances a process known as ER associated degradation (ERAD), which directs misfolded proteins for destruction by the cytoplasmic ubiquitin-proteosome pathway [Römisch, 2005]. ERAD can be divided into separate steps, which includes detection of misfolded protein in the ER lumen, a step that requires molecular chaperones, for example, GRP78/BiP, transport of the proteolytic substrates to the cytosol, polyubiquitin, and digestion in the cytosolic 26S proteasome. Proteosomal activity in islets is effectively inhibited by lactacystin [Kitiphongspattana et al., 2005]. Co-treatment of INS-1E cells with both thapsigargin and lactacystin did to some extent reverse the thapsigargin-induced reduction in GRP78/BiP levels. However, the interpretation of these findings is obscured by the fact that lactacystin alone tended (albeit not significant) to increase GRP78/BiP levels. Therefore, our results do not allow us to conclude that proteosomal degradation of GRP78/BiP alone can explain the full reduction in GRP78/BiP seen after thapsigargin treatment. Yet another alternative pathway for cellular protein degradation is autophagy, a process in which cells degrade misfolded proteins in polymers or aggregates [Ding and Yin, 2007]. An investigation of pancreata from donors with a previous history of T2D showed signs of altered autophagy, indicating that disturbances in this system might be part of type T2D pathogenesis [Masini et al., 2009]. An important step in the process of autophagy is the formation of the autophagosome, where Apg5 (the mouse homologue to Atg5 in yeast) plays an important role [Hara et al., 2006]. A hallmark of autophagy is the conversion of a pre-lipidated form of LC3B-I to its lipidated form LC3B-II [Ogata et al., 2006]. We took a pharmacological approach to inhibit the formation of autophagosomes using 3-MA [Fujimoto et al., 2009]. 3-MA partially inhibited the thapsigargin-induced conversion of LC3B-I to LC3B-II. In addition, 3-MA showed antagonizing effects on thapsigargin-induced reduction of GRP78/BiP.

Another interesting aspect is the lack of increase (rather than the actual decrease) of chaperone expression. An adaptive chaperone response to ER stress would result in increased expression of GRP78/BiP. However, in the literature there are numerous examples where this does not occur, in both β-cells [Karaskov et al., 2006] and islets [Elouil et al., 2007], and our article adds to these observations. In an additional report [Matveyenko et al., 2009] compared a standard chow versus high-fat diet in wild-type rats and in rats over-expression human islet amyloid polypeptide (hIAPP). In the wild-type rat model high-fat diet induced GRP78/BiP expressing, occurring alongside β-cell adaptation and maintained normoglycaemia. In contrast in the hIAPP over-expressing rats a high-fat diet seemed to decrease GRP78/BiP levels and indeed these rats became diabetic. Thus failure to mount an adaptive chaperone response is indeed physiological important and adds argument to develop pharmacological tools such chemical chaperones [Özcan et al., 2006].

Taken together, our findings indicate that thapsigargin decreases GRP78/BiP, PDIA3, and PDIA6 protein levels in INS-1E cells. Investigation of GRP78/BiP reveals that the decrease is not a consequence of reduced mRNA expression, but appears to involve both reduction in protein synthesis and enhanced degradation via both the proteosome and autophagy. The mechanisms for the enhanced decrease of the PDIs by thapsigargin were not fully examined in the present study.

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