GRP78, but Not Protein-disulfide Isomerase, Partially Reverses Hyperglycemia-induced Inhibition of Insulin Synthesis and Secretion in Pancreatic β-Cells*§

Liling Zhang†, Elida La†§, Tracy Teodoro§†, and Allen Volchuk§†‡

From the †Division of Cell and Molecular Biology, Toronto General Research Institute, University Health Network, Toronto, Ontario MSG 1L7, Canada and the Departments of §Biochemistry and §Physiology, University of Toronto, Toronto, Ontario MSS 1A8, Canada

Chronic hyperglycemia contributes to pancreatic β-cell dysfunction during the development of type 2 diabetes. Treatment of pancreatic β-cells with prolonged high glucose concentrations has been shown to reduce insulin promoter activity and insulin gene expression. Here, we examined the effect of high glucose on endoplasmic reticulum (ER) stress pathway activation and insulin production in INS-1 832/13 pancreatic β-cells. Treatment of cells with 25 mM glucose for 24–48 h decreased insulin mRNA and protein levels and reduced the proinsulin translation rate, which was accompanied by enhanced unfolded protein response pathway activation (XBP-1 mRNA splicing and increased phospho-eIF2α, CHOP, and active ATF6 levels). Overexpressing the ER chaperone GRP78 partially rescued high glucose-induced suppression of proinsulin levels and improved glucose-stimulated insulin secretion with no effect on insulin 2 mRNA levels. Under these conditions, there was little effect of GRP78 overexpression on ER stress markers. Knockdown of GRP78 expression under basal glucose conditions reduced cellular insulin levels and glucose-stimulated insulin secretion. Thus, GRP78 is essential for insulin biosynthesis, and enhancing chaperone capacity can improve β-cell function in the presence of prolonged hyperglycemia. In contrast, overexpression of the ER chaperone and oxidoreductase protein-disulfide isomerase (PDI) reduced glucose-stimulated insulin secretion and induced ER stress resulting from the accumulation of proinsulin in the ER. These results suggest a role for both GRP78 and PDI in insulin biosynthesis, although an excess of PDI disrupts normal proinsulin processing.

Type 2 diabetes is associated with various abnormalities, including insulin resistance, dyslipidemia, and pancreatic β-cell dysfunction (1). The disease is characterized by abnormally elevated blood glucose levels. Prolonged or chronic hyperglycemia has been implicated in causing or contributing to detrimental effects in various tissues, such as the kidney, eye, peripheral nerves, and pancreatic β-cells, that lead to diabetic complications and exacerbation of the disease.

In pancreatic β-cells, an acute increase in blood glucose normally stimulates insulin secretion, increases proinsulin translation, and enhances insulin gene transcription (2). However, prolonged hyperglycemia has been shown to cause various detrimental effects on insulin secretion and insulin biosynthesis (3–5). Chronic high glucose reduces glucose-stimulated insulin secretion and the function of several key transcription factors, such as Pdx-1 and MafA, resulting in reduced insulin gene transcription. In addition, prolonged hyperglycemia may have effects on proinsulin translational control, although this still requires further study (6, 7). These detrimental effects of prolonged high glucose in β-cells have been suggested to be mediated, at least in part, from increased cellular reactive oxygen species (8, 9).

In addition, prolonged high glucose exposure has been proposed to induce endoplasmic reticulum (ER) stress in β-cells (10–13). ER stress occurs when the protein folding capacity of the ER is not sufficient to deal with protein folding demands or when an excess of misfolded or aggregated proteins accumulate. Such conditions activate the unfolded protein response (UPR) that transiently reduces the amount of new protein synthesis and increases folding capacity and degradation of terminally misfolded proteins (14). ER stress is physiologically encountered by the pancreatic β-cell, which must regulate its chaperone capacity to meet normal insulin biosynthetic demands. Insulin translation is regulated in the β-cell via eIF2α phosphorylation by the PERK pathway (15), and this pathway is critical to β-cell function. Knock-out of PERK leads to severe β-cell dysfunction and death in rodents (16, 17), and mutations in this gene cause diabetes in humans as a result of β-cell failure (18).

The consequences of prolonged ER stress potentially due to chronic hyperglycemia or the contribution that ER stress has in

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‡ Recipient of a Tier II Canada Research Chair award. To whom correspondence should be addressed: Toronto General Research Institute, University Health Network, 101 College St., TMDT 10-707, Toronto MSG 1L7, Ontario, Canada. Tel: 416-581-7675; E-mail: avolchuk@uhnres.utoronto.ca.

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The abbreviations used are: ER, endoplasmic reticulum; UPR, unfolded protein response; PDI, protein-disulfide isomerase; GFP, green fluorescent protein; PDIm, PDI mutant; siRNA, small interfering RNA; RT, reverse transcription; ANOVA, analysis of variance; PBA, sodium 4-phenylbutyrate.
causing abnormal effects on β-cell function (insulin biosynthesis) or survival are not fully established. In this study, we examined the effect of prolonged high glucose on ER stress pathway activation and insulin production and secretion in INS-1 832/13 pancreatic β-cells. Treatment of INS-1 832/13 cells with prolonged high glucose decreased insulin mRNA and protein levels. Although some markers of UPR signaling were elevated, it appears that hyperglycemia per se does not elicit a characteristic ER stress response as chaperone expression was not induced by high glucose. However, we found that GRP78 and protein-disulfide isomerase (PDI), two ER chaperone proteins, are required for normal insulin synthesis. Overexpressing GRP78 partially rescued prolonged high glucose-induced suppression of steady-state proinsulin levels and suppression of glucose-stimulated insulin secretion.

**EXPERIMENTAL PROCEDURES**

**Recombinant Adenovirus Production**—Recombinant adenoviruses expressing full-length human GRP78 or green fluorescent protein (GFP) were prepared as described (19). Full-length human PDI cDNA was amplified by PCR from pCMV6-SPORT, a plasmid obtained from Dr. Lloyd Ruddock (University of Oulu, Finland), and cloned into pCRII-TOPO vector (Invitrogen). The resulting TOPO-PDI was cut with EcoRI and cloned into the EcoRI site of the pEGFP-N1 vector (BD Biosciences). Cloning of the full-length PDI into pShuttle-IRES-hrGFP-2 vector (Stratagene) was performed using the BglII/SalI sites. For production of PDI mutant construct (PDIm), the cysteine residues in the catalytic thioredoxin-like domains of PDI (Cys-53, Cys-56, Cys-397, and Cys-400) were mutated to serine using the QuikChange II XL site-directed mutagenesis kit (Stratagene).

To produce an adenovirus expressing GRP78, PDI, or enhanced GFP, the AdEasy XL adenoviral vector system (Stratagene) was used according to the instructions provided. After preparing the primary viral stock by freeze-thawing infected AD-293 cells, the viral particles were subjected to one round of amplification. The amplified adenovirus was then used to infect four 10-cm plates of AD-293 cells and was purified using the Vivapure AdenoPACK 100 kit (Vivascience). The purified virus was resuspended in buffer (20 mM Tris/HCl, 25 mM NaCl, 2.5% glycerol, pH 8.0), and aliquots were stored at −80 °C. The viral titer was determined by measuring the absorbance at 260 nm.

**INS-1 832/13 Cell Culture and Infection**—Rat INS-1 832/13 pancreatic β-cells were obtained from Dr. Chris Newgard (Duke University) and were grown as described previously (20). INS-1 832/13 cells (300,000 cells/well in a 12-well plate) were infected with 9 × 10^8 pfu/ml of Ad-GRP78, Ad-PDI, Ad-PDIm, or Ad-GFP and incubated at 37 °C in 5% CO_2 for 2 h with gentle shaking every 30 min. The cells were then washed once with phosphate-buffered saline and replaced with fresh RPMI 1640 medium. After culturing for 24 h the cells were treated as indicated in the figure legends. Control cells were cultured under the same conditions without the addition of adenovirus.

**siRNA Transfection**—INS-1 832/13 cells were transfected with GRP78 siRNA (Invitrogen) or control GFP siRNA (obtained from Dr. Ouathek Ouerrelli, Sloan-Kettering Institute, New York) using Lipofectamine RNAiMAX reagent (Invitrogen) according to the manufacturer’s instructions. Briefly, per transfection 12 pmol of siRNA was diluted in 200 μl of Opti-MEM medium without serum (Invitrogen) directly in one well of a 12-well plate and mixed gently. After incubation at room temperature for 5 min, 2 μl of Lipofectamine RNAiMAX reagent (Invitrogen) was added to each well containing the diluted siRNAs, mixed gently, and incubated at room temperature for 20 min. During the incubation period, cells were diluted in medium without antibiotics to obtain a concentration of 100,000 cells/ml. To each well containing siRNA/Lipofectamine RNAiMAX complexes, 1.0 ml of the diluted cells was added (i.e. 100,000 cells/well) to obtain a final siRNA concentration of 10 nm. The cells were mixed gently and incubated at 37 °C for 72 h. The cells were treated as indicated in the figure legends and processed for either Western blot analysis or insulin radioimmunoassay.

**Insulin Secretion Assay**—Insulin secretion assays were performed after 24 h of adenoviral infection and 24 h of treatment with 5 or 25 mM glucose in RPMI 1640 medium. INS-1 832/13 cells were washed once with 2 ml of glucose-free KRbH buffer (128.8 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM MgSO_4, 2.5 mM CaCl_2, 5 mM NaHCO_3, 10 mM Hepes, 0.1% bovine serum albumin, pH 7.4) and then incubated in glucose-free KRbH buffer for 1 h. The cells were then washed once with KRbH and incubated with 1 ml of KRbH (2.8 mM glucose; basal) or KRbH (15 mM glucose; stimulated) for 1 h. Following the 1-h incubation, the culture plate was placed on ice, and the medium was collected for insulin radioimmunoassay (described below).

The cells were lysed in ice-cold lysis buffer (1% Triton X-100, 20 mM HEPES, pH 7.3, 100 mM KCl, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor mixture (Roche Applied Science)). Cells were lysed on ice for 30 min and centrifuged at 13,000 rpm for 10 min at 4 °C. The supernatant was then transferred to a new tube, and the protein concentration was measured using a BCA protein assay (Pierce). For insulin assays, the culture media were centrifuged at 5500 rpm for 5 min at 4 °C, and insulin present in the supernatant was measured using a rat insulin radioimmunoassay kit according to the manufacturer’s instructions (Linco Inc.).

**[35S]Methionine Labeling and Proinsulin Immunoprecipitation**—For Fig. 1C, INS-1 832/13 cells were seeded in a 6-well plate (700,000 cells/well) the day before the experiment. The cells were then incubated in 5 or 25 mM glucose for 24 h. Cells were then washed with phosphate-buffered saline and incubated in 5 or 25 mM glucose in the presence of 100 μCi/ml of Tran35S-label (MP Biomedicals) in 1-methionine-free RPMI 1640 medium for 1, 3, and 5 h at 37 °C. At the indicated time points, the cells were washed twice with phosphate-buffered saline and lysed in ice-cold 1% Triton X-100 lysis buffer (1% Triton X-100, 20 mM HEPES, pH 7.4, 100 mM KCl, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin) for 40 min at 4 °C. The lysates were then centrifuged at 13,000 rpm for 10 min at 4 °C, and 60 μg of the cleared lysate was used for immunoprecipitation using goat polyclonal anti-rat C-peptide antibody (Linco Research Inc.) or control rabbit IgG serum. The proteins were resolved by a 4–12% NuPage gel (Invitrogen). The dried gel was exposed
overnight at −80 °C to photographic film (Eastman Kodak Co. BioMax Light).

RNA Isolation and Real Time PCR Analysis—Total RNA was isolated from INS-1 832/13 cells using TRIzol reagent (Invitrogen). The RNA was reverse transcribed to single-stranded cDNA using the High-Capacity cDNA reverse transcription kit (Applied Biosystems). The resulting cDNA was used for real time PCR analysis using the TaqMan Gene Expression system (Applied Biosystems). Gene-specific primers were obtained from Applied Biosystems: rat GRP78 (Rn01435771_g1), PDI (Rn00564459_ml), ATF4 (Rn00824644_g1), CHOP (Rn00492098_g1), Sel1 (Rn00710081_ml), EDEM1 (Rn01421307_ml), and insulin 2 (Rn01774648_g1). The β-actin-specific primers (rat β-actin) and TaqMan MGB probes were also obtained from Applied Biosystems. Serial dilutions of INS-1 c cDNA were used to generate a standard curve. A complete real time PCR reaction (25 µl) contained 10 µl of cDNA, 1.25 µl of double-distilled H2O, 1.25 µl of TaqMan gene expression primers (20×), and 12.5 µl of TaqMan Universal PCR Master Mix (2×). Samples were loaded into an ABI PRISM 96-well optical reaction plate. Reactions were run on an ABI Prism 7900HT sequence detection system using the following protocol: 10 min at 95 °C, 40 cycles of 15 s at 95 °C, and 1 min at 60 °C. The standard curve and corresponding values from each sample were determined by the SDS 2.1 software of the ABI Prism 7900HT instrument. Values were normalized to expression of β-actin mRNA for each species and presented as a mean ± S.E. of a minimum of three independent experiments.

XBP-1 mRNA Splicing—Total RNA was isolated from INS-1 832/13 cells as described above. Rat XBP-1 cDNA was amplified by RT-PCR (Qiagen OneStep RT-PCR kit) using primers that flank the intron excised by IRE1 exonuclease activity as described previously (21). Primer sequences used to amplify rat XBP-1 were 5′-AAA CAG AGT AGC ACA GAC GAC TGCC-3′ and 5′-TCC TTC TGG GTA GAC CTC TGG GAGG-3′. The protocol used for the RT-PCR was as follows: 50 °C (30 min); 95 °C (15 min); 30 cycles of 94 °C (1 min), 62 °C (1 min), 72 °C (1 min); 72 °C (10 min). RT-PCR products were resolved on a 3% agarose gel and visualized using ethidium bromide.

Trichloroacetic Acid Protein Precipitation and Western Blot Analysis—For the experiments in Fig. 9, cells were washed with phosphate-buffered saline on ice, and 9% ice-cold trichloroacetic acid (200 µl/well) was added. The cells were scrapped into tubes and centrifuged at 13,000 rpm for 10 min at 4 °C. The pellet was washed with 1 ml of cold acetone and centrifuged at 13,000 rpm for 10 min at 4 °C. The pellet was resuspended in 2× LDS sample buffer (Invitrogen) without reducing agent.

Proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Following incubation with secondary antibody conjugated to horseradish peroxidase, the bands were detected with the enhanced chemiluminescence system (Amersham Biosciences). Immunoblots were scanned and quantified using Scion Image software. For detection of insulin by immunoblotting, the samples were resolved using 4–12% NuPage gels (Invitrogen). The following commercial primary antibodies were used: anti-KDEL (SPA-827, 1:1000; StressGen), PDI (SPA-890, 1:4000; StressGen), anti-phospho-eIF2α (catalog number 9721, 1:500; Cell Signaling), γ-tubulin (T6557, 1:1000; Sigma), rabbit anti-insulin (sc-9168, 1:250; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-α-actin (CSA-400, 1:1000; StressGen), and GM130 (G65120, 1:500; Transduction Laboratories).

The rabbit anti-ATF6 antibody was generated by Covance Research Inc. to a peptide sequence in the cytosolic portion of ATF6 (SENQRKVPSPKR) conjugated to keyhole limpet hemocyanin. This sequence is conserved between rodents and human ATF6. The terminal bleeds from two rabbits were pooled, and the antibody was affinity-purified by passing the serum through a column containing human GST-ATF6 cytosolic domain (amino acids 1–374) covalently coupled to glutathione-agarose beads. The antibody was eluted in glycine-HCl (pH 2.5) and neutralized with phosphate buffer.

Data Analysis—Results are presented as mean ± S.E. Statistical significance was analyzed by Student’s two-sample t test assuming equal variance or ANOVA followed by Tukey’s test. p < 0.05 was considered statistically significant.

RESULTS

Prolonged Hyperglycemia Reduces Steady-state Proinsulin Levels and Increases ER Stress—Prolonged or chronic hyperglycemia is known to cause several adverse effects in pancreatic β-cells, including blunting glucose-stimulated insulin secretion and inhibiting insulin gene transcription (8, 9). These effects can be observed in cultured pancreatic β-cells, which have been used as models to study the effects of prolonged high glucose in vitro (13, 22–24). We have confirmed these results using the INS-1 832/13 β-cell line. High glucose treatment for 24–48 h markedly reduced steady-state proinsulin and insulin protein levels (Fig. 1A) and insulin 2 mRNA levels (Fig. 1B). We also

![Figure 1](image-url)
observed that proinsulin translation was reduced in cells exposed to chronic high glucose by measuring the incorporation of \[^{35}S\]methionine into proinsulin immunoprecipitated using an anti-C-peptide antibody (Fig. 1C).

We next examined whether prolonged high glucose induces ER stress by monitoring UPR pathway signaling and the levels of downstream target genes. Prolonged high glucose exposure increased the levels of spliced XBP-1 mRNA (an indicator of IRE-1 activation) (Fig. 2A), and the levels of phosphorylated eIF2\(\alpha\), indicative of PERK activation (Fig. 2B). We also monitored the levels of cleaved ATF6-p50 using an antibody generated to the cytosolic portion of ATF6. This antibody recognizes overexpressed cleaved (active) ATF6-p50, and endogenous levels are increased by pharmacological inducers of ER stress (see Fig. S1). The levels of ATF6-p50 detected by this antibody were higher in INS-1 832/13 cells grown in prolonged high glucose conditions compared with control cells, but the difference was modest (Fig. 2C). Some downstream genes known to be induced by the UPR were increased by chronic hyperglycemia, including mRNA levels of ATF4 and CHOP (GADD153) (Fig. 2D). However, chaperone genes that are normally induced by ER stress (GRP78 and PDI) and genes associated with ERAD (SEL1 and EDEM1) were not affected or actually decreased by prolonged hyperglycemia (Fig. 2D). Tunicamycin, an inhibitor of N-linked glycosylation, increased the levels of these proteins, as expected. Thus, it appears that some genes that would normally be induced by ER stress are not increased under hyperglycemic conditions. Combined, these results indicate that the UPR pathways are activated in cells that are cultured in prolonged hyperglycemic conditions, although not all downstream target genes are induced.

Overexpression of GRP78 Increases Steady-state Proinsulin Levels and Improves Glucose-stimulated Insulin Secretion—Prolonged high glucose may perturb normal ER function, which could contribute to detrimental effects on insulin biosynthesis. ER stress is sensed both by the detection of unfolded proteins directly and by GRP78 interaction with ER stress sensors (14). We therefore examined whether manipulating cellular GRP78 levels could affect insulin biosynthesis and secretion in the presence of prolonged hyperglycemia.

We overexpressed GRP78 in INS-1 832/13 cells by adeno viral transduction and monitored the effect on proinsulin and insulin levels in control cells and cells exposed to prolonged high glucose. In these experiments, we also expressed a control protein GFP and the ER chaperone and oxido reductase PDI by adenovirus transduction and monitored the effect on proinsulin and insulin levels in control cells and cells expressing the various proteins. However, in cells overexpressing GRP78 or PDI, the levels of proinsulin in both control (5 mM) and high glucose-treated cells was higher compared with GFP-expressing or uninfected cells (Fig. 3, A, B, and E). Interestingly, overexpression of PDI increased the steady-state intracellular proinsulin levels to a greater extent than overexpression of GRP78, particularly under high glucose conditions. Thus, it appears that increasing chaperone capacity can increase the amount of cellular proinsulin even in the face of prolonged hyperglycemia. The increase in steady-state proinsulin levels is likely to occur at either the translational level or enhanced protein folding, since the reduction in insulin mRNA in response to prolonged high glucose exposure was not affected by either GRP78 or PDI overexpression (Fig. 4).

We also examined if overexpressing GRP78 affected insulin secretion in INS-1 832/13 cells. Cells were infected and kept at low (5 mM) glucose for 24–48 h, after which 1-h static glucose-stimulated secretion experiments were performed at basal (2.8 mM) and stimulatory (15 mM) glucose concentrations. Under these experimental conditions, we observed an ~10-fold
increase in insulin secreted into the media at stimulatory glucose, but there was no difference in insulin secretion between control cells (uninfected or GFP-infected) and GRP78 overexpressing cells (results not shown). Control and infected cells were also pretreated with hyperglycemic conditions (25 mM glucose) for 24 h prior to insulin secretion experiments. Under these conditions glucose-stimulated insulin secretion was markedly reduced (<2-fold 15 mM glucose versus 2.8 mM glucose). However, cells overexpressing GRP78 had significantly better glucose-stimulated insulin secretion compared with controls (Fig. 5). The -fold difference in insulin secretion between stimulated (15 mM) and basal (2.8 mM) glucose was 2.0 ± 0.27 in GRP78-expressing cells compared with 1.51 ± 0.14 in GFP-expressing cells. Thus, enhancing chaperone capacity by increasing GRP78 levels increases proinsulin levels and glucose-stimulated insulin secretion in the face of prolonged hyperglycemia. Unexpectedly, insulin secretion at basal glucose and under glucose-stimulated conditions was markedly reduced in PDI-overexpressing cells (Fig. 5).

Protein folding can be enhanced by small molecule chemical chaperones, such as sodium 4-phenylbutyrate (PBA) (25). The presence of PBA, but not an antioxidant, N-acetylcysteine, increased proinsulin expression, showing that improving chaperone capacity can enhance steady-state proinsulin levels under hyperglycemic conditions (Fig. 6). Although PBA clearly increased steady-state proinsulin levels, there was no significant effect on cellular processed B-chain insulin levels, as measured by Western blotting. In fact, B-chain insulin levels were reduced in PBA-treated cells under basal glucose levels (Fig. 6A).

We also examined the effect of reducing endogenous GRP78 expression on insulin levels and secretion in cells grown under basal glucose conditions. Knocking down endogenous GRP78 expression by >50% resulted in a marked reduction in steady-state proinsulin and processed insulin levels (Fig. 7A) and reduced basal and glucose-stimulated insulin secretion (Fig. 7B). Thus, GRP78 is critical for maintaining normal insulin levels and secretion in INS-1 832/13 β-cells.

Since overexpression of GRP78 reduces the effects of prolonged hyperglycemia on proinsulin levels and insulin secretion, we examined if GRP78 overexpression also reduces ER stress. However, under the experimental conditions tested (24 or 48 h of high glucose exposure) we were unable to detect any significant differences in XBP-1 splicing (i.e. XBP-1 splicing still occurred in GRP78-overexpressing cells) (results not shown). In addition, overexpressing GRP78 failed to reduce CHOP mRNA levels induced by prolonged high glucose exposure (see Fig. 10B). Thus, GRP78 overex-
expression is not sufficient to completely relieve ER stress in the continued presence of high glucose.

Overexpression of PDI Alters Normal Proinsulin Disulfide Bond Formation and Induces Some Markers of ER Stress—Unexpectedly, despite increasing steady-state proinsulin levels (Fig. 3), overexpressing PDI markedly reduced insulin secretion (Fig. 5). We surmised that overexpression of PDI may alter the normal balance of disulfide bond formation and possibly impede proinsulin folding. We examined this hypothesis by overexpressing a mutant version of PDI that had the four cysteine residues in the Cys-X-X-Cys motifs in the two catalytically active a and a’ domains mutated to serine residues (26). These cysteine residues are critical for disulfide bond catalysis, and mutation of these residues renders PDI inactive (26). Overexpression of the mutant PDI, unlike wild-type PDI, did not result in an increase in the steady-state levels of proinsulin (Fig. 8, A and B). Furthermore, overexpression of the mutant PDI had no effect on insulin secretion (Fig. 8 C). Thus, PDI catalytic activity is required to elicit the effects on proinsulin levels and insulin secretion observed.

As part of the mechanism of PDI-catalyzed disulfide bond formation, PDI covalently binds transiently to client proteins via disulfide bonds (26, 27). Thus, overexpression of PDI may shift the equilibrium of PDI to more mixed disulfides, consequently preventing efficient disulfide bond formation on client proteins. To test this, we prepared cell extracts of control and PDI-overexpressing cells under nonreducing conditions and immunoblotted the extracts with anti-PDI and anti-insulin antibodies. Under nonreducing conditions, a band was observed at the expected Mr for PDI as well as a higher Mr smear that was prominent only in cells overexpressing PDI, as detected with the PDI antibody (Fig. 9 A, top). Little proinsulin was detected migrating at the expected size for reduced proinsulin (Fig. 9 A, bottom). Under reducing conditions, however, the higher Mr smear was largely absent, and a band of the expected Mr for proinsulin was observed (Fig. 9 A). The high Mr,
DISCUSSION

Chronic hyperglycemia is known to cause abnormal insulin biosynthesis and glucose-stimulated insulin secretion in pancreatic β-cells, which is believed to contribute to β-cell dysfunction in diabetes (8). The INS-1 832/13 pancreatic β-cell line has been used as a model system to study the effects of chronic hyperglycemia, since the cell line is cultured in mildly hyperglycemic conditions (11 mM), and exposure to high glucose (25–30 mM) for several days induces several adverse effects on β-cell function (13, 22–24). As we show here, exposure of INS-1 832/13 cells to prolonged high glucose results in a marked reduction in steady-state cellular insulin protein and insulin mRNA levels and a marked diminution of glucose-stimulated insulin secretion.

The mechanisms by which prolonged high glucose causes these abnormalities are not fully characterized. In this study, we examined whether chronic hyperglycemia induces ER stress and whether ER stress may contribute to the detrimental effects of high glucose on insulin synthesis and secretion in INS-1 832/13 β-cells. ER stress induces the UPR, which initiates signals that improve ER function by increasing folding capacity and increasing the degradation of unfolded proteins in the ER. We observed that prolonged high glucose caused ER stress, since all three UPR signaling pathways were activated to some degree. Prolonged high glucose induced XBP-1 mRNA splicing and increased the levels of phosphorylated eIF2α and active ATF6p50. These results are consistent with recent studies that have shown similar effects in response to high glucose in INS-1 cells (11, 12, 28) and rat islets (10). How chronic high glucose causes ER stress is not established, although high glucose is known to induce oxidative stress, which can also cause ER stress (29).

Interestingly, despite measurable activation of the UPR pathways by prolonged hyperglycemia, there was a differential induction of target genes. An increase in the levels of ATF4 and CHOP mRNA was observed. However, other known target genes including chaperone proteins (PDI and GRP78) and ERAD components (SEL1 and EDEM1) were not induced by high glucose under our experimental conditions. The reason for this is not known, although it is possible that activation of UPR pathways is not sufficient to induce significant downstream gene expression changes, or the pathways may not signal normally. These results suggest that the ER stress response may be impaired under hyperglycemic conditions. Alternatively, this may be a feature of this cell line, since rat islets cultured in high glucose for 18 h induced a small but detectable increase in UPR target genes, such as GRP78 and EDEM (10).

GRP78 protein levels were also not induced by prolonged high glucose conditions in the INS-1 832/13 cell line. We surmised that overexpression of GRP78 would enhance the ability of β-cells to deal with misfolded proteins and improve β-cell function under hyperglycemic conditions. Overexpression of
GRP78 has been shown to increase ER stress resistance and have beneficial effects in several cell types (19, 30–32). Indeed, we found that the levels of cellular proinsulin were increased, and glucose-stimulated insulin secretion was significantly improved in cells overexpressing GRP78. The effect on proinsulin levels could be due to increased biosynthesis or more likely enhanced folding, since there was no effect on insulin mRNA levels. A similar result was obtained using the chemical chaperone PBA, indicating that GRP78 probably enhances proinsulin protein folding. These results are consistent with a recent study that showed that PBA can improve glucose-stimulated insulin secretion in INS-1 cells exposed to palmitate (33).

In addition, we found that depleting endogenous GRP78 levels leads to a significant reduction in cellular proinsulin levels as well as insulin secretion. Combined, our results show that GRP78 is vital for normal insulin biosynthesis, and increasing GRP78 levels has beneficial effects on pancreatic β-cell function.

The ER-localized protein GRP78 is a multifunctional molecular chaperone protein that is also involved in controlling the activation of the UPR pathways in response to ER stress (14). Thus, we expected that the significant improvement in proinsulin levels and glucose-stimulated secretion under hyperglycemic conditions by overexpression of GRP78 might be due to reduced ER stress. However, GRP78 overexpression did not significantly affect hyperglycemia-induced XBP-1 splicing or CHOP mRNA levels. This indicates that ER stress may not be prevented by GRP78 overexpression under the experimental conditions used. The reason for this is not known, although it is possible that the increased levels of overexpression achieved were not sufficient to completely restore normal ER function in the face of prolonged hyperglycemia. Activation of IRE-1 by high glucose exposure has been suggested to lead to insulin mRNA degradation by the endonucleolytic activity of IRE-1 (28, 34). The hyperglycemia-induced reduction in insulin 2 mRNA levels was not affected by overexpressing GRP78, further supporting the observation that ER stress was still persistent in these cells.
Although it appears that overexpressing GRP78 failed to fully prevent ER stress, our results suggest that GRP78 is essential for insulin biosynthesis and folding and that enhancing chaperone capacity can improve insulin levels and β-cell function even under diabetic conditions. Improving protein folding either by overexpression of the ER chaperone oxygen-regulated protein or using chemical chaperones has been shown to improve insulin resistance in diabetic models (35, 36). It would be interesting to examine whether targeted overexpression of GRP78 specifically in β-cells leads to similar beneficial effects in vivo.

In contrast to overexpressing GRP78, overexpression of the abundant and ubiquitously expressed ER chaperone and oxidoreductase PDI resulted in increased proinsulin levels but reduced insulin secretion. This effect can be explained by the fact that increasing PDI expression leads to an accumulation of proinsulin that forms covalent mixed disulfides with PDI itself and possibly with other oxidoreductases (Fig. 9). These effects are not observed with the PDI mutant lacking the catalytic cysteine residues. The molecular mechanism of disulfide bond formation has been delineated in some detail with the recent identification and structure of Ero1 oxidase (37). The pathway involves transfer of oxidizing equivalents from Ero1 to PDI and from PDI to secretory proteins through direct thiol-disulfide exchange reactions. Thus, by increasing the levels of PDI/proinsulin, it appears that this shifts the balance of PDI toward prolonged interaction with proinsulin. PDI has an isomerization function and probably samples secretory proteins continuously while they are still in the ER. As a result, overexpression of PDI probably increases the levels of misfolded proinsulin in the ER and induces some ER stress (Fig. 10). Interestingly, we detected minimal IRE-1 pathway activation (XBP-1 splicing), indicating that not all of the UPR pathways are efficiently activated by PDI overexpression. Our interpretation that PDI overexpression causes a build up of proinsulin in the ER is consistent with a study that examined the effect of PDI overexpression on secretory protein transport in CHO cells. Overexpression of PDI decreased the secretion of an overexpressed disulfide-bonded protein (TNFR) but not a protein lacking disulfides (38).

There have been some in vitro studies showing that PDI can act on proinsulin to promote disulfide bond formation (39, 40). To our knowledge, this is the first report that shows that PDI affects proinsulin disulfide bond formation in living cells. Interestingly, the levels of PDI are reduced in cultured MIN6 cells (15, 41). This observation is consistent with the finding that increasing PDI expression results in significantly reduced insulin levels and insulin secretion (16). We also discovered that PDI is involved in insulin disulfide bond formation and that an excess of this protein impedes normal insulin folding and probably exit from the ER. Enhancing GRP78 expression or function may be a rational strategy for improving β-cell function in treating diabetes.

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GRP78 and Insulin Production and Secretion
GRP78 and Insulin Production and Secretion

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