Proteasome Inhibition Alters Glucose-stimulated (Pro)insulin Secretion and Turnover in Pancreatic β-Cells*

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Metabolic labeling studies were conducted in freshly isolated mouse islets and a β-cell line (MIN6) to examine the effects of proteasome inhibition on glucose-stimulated (pro)insulin synthesis and secretion. Glucose-stimulated (pro)insulin synthesis, as determined by the incorporation of [3H]tyrosine, decreased significantly by 90% in islets and 71% in MIN6 cells pretreated with the proteasome inhibitor lactacystin (10 μM) for 2 h. To follow the fate of newly synthesized (pro)insulin, islets were pulse-labeled with [3H]tyrosine (40 μCi) for 20 min and chased ± lactacystin (10 μM) for up to 4 h. The release of newly synthesized (pro)insulin ([3H]tyrosine-labeled) was similar between lactacystin-treated and control islets despite a 51% decrease (p < 0.05) in total immunoreactive (pro)insulin secretion by lactacystin-treated islets. The specific radioactivity of [3H]tyrosine-labeled (pro)insulin in the extracellular medium of lactacystin-treated islets (0.52 ± 0.16 cpm/microunits) was 2-fold greater relative to control islets (0.25 ± 0.06 cpm/microunits). Induction of the unfolded protein response by lactacystin, as evidenced by the up-regulation of endoplasmic reticulum (ER) chaperones (GRP78/BiP, GRP94, protein disulfide isomerase) and induction of the stress-inducible transcription factor C/EBP-homologous protein/GADD153 (CHOP/GADD153), likely contributed to the release of newly synthesized (pro)insulin to relieve ER stress. The present data indicate proteasome inhibition did not prevent, but increased (p < 0.05), the intracellular degradation of [3H]tyrosine-labeled (pro)insulin from 8 to 24% in islets. Collectively, these data indicate β-cells may balance glucose-stimulated (pro)insulin synthesis and secretion with the activity of the proteasome to regulate protein concentrations in the ER.

Over two decades ago it was hypothesized that a significant proportion of newly synthesized proteins do not attain their native structure owing to errors in translation or defects in post-translational protein folding (1). This was confirmed by Schubert et al. (2) who demonstrated that upwards of 30% of newly synthesized “non-functional proteins,” described as defective ribosomal products, may be polyubiquitinated and rapidly degraded by the proteasome (2, 3). Therefore, the efficiency and fidelity of overall protein biogenesis are dependent on cellular proteasome activity. The proteasome also contributes to endoplasmic reticulum (ER)–associated degradation as part of the unfolded protein response (UPR), which relieves ER stress. At least three functionally distinct responses comprise the UPR (reviewed in Refs. 4–6): 1) transcriptional up-regulation of genes encoding ER chaperones; 2) translational attenuation to reduce protein influx into the ER and further accumulation of misfolded proteins; and 3) induction of ER-associated degradation (ERAD) components. Through these mechanisms, the mammalian UPR seems to coordinate both transcriptional and translational controls involved in cell survival, inflammation, immune responsiveness, and apoptosis (5–8).

Protein quality control and clearance mechanisms, such as the ubiquitin–proteasome system, are especially important in pancreatic β-cells, whose function is to synthesize and secrete biologically active insulin. Indeed, the relevance of ER quality control to diabetes has been revealed by two diabetic mouse models (6). In inbred C57BL/6-Ins2Akita mice, an autosomal dominant missense mutation of the Ins2 gene leads to blockage of proinsulin transport from the ER to the Golgi, β-cell dysfunction, and diabetes (9, 10). In addition, a close correlation was observed between the effects of aging on the loss of β-cell mass and the development of hyperglycemia in the Perk−/− mouse, which lacks the protein kinase, PERK (PKR-like ER kinase, also known as PEC; formally eukaryotic translation initiation factor 2α kinase 3), whose function links protein folding and polypeptide translation (11, 12). It is thought that the lack of PERK leads to a failure in translational attenuation following activation of ER stress response genes (11–13).

Despite the apparent relevance of protein quality control to diabetes, the role of the proteasome in insulin turnover or any other vital β-cell function has received limited attention (14, 15). The present studies examined the effects of proteasome inhibition on glucose-stimulated mouse islets and pancreatic MIN6

1 The abbreviations used are: ER, endoplasmic reticulum; BiP, binding protein; BSA, bovine serum albumin; CHOP, C/EBP-homologous protein; ERAD, ER-associated degradation; GADD153, growth arrest and DNA-damage-inducible gene 153; GRP, glucose-regulated protein; ins1/2, insulin 1/2; PERK, PKR-like ER kinase; PDI, protein disulfide isomerase; qRT-PCR, quantitative reverse-transcriptase PCR; UPR, unfolded protein response; PBS, phosphate-buffered saline; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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cells. Experimental data and a working model are presented to account for the observed alterations in intracellular (pro)insulin turnover and secretion in proteasome-inhibited \( \beta \)-cells.

**EXPERIMENTAL PROCEDURES**

**Mouse Islet Isolation and Cell Culture**—Mouse islets were isolated from female (phenotypically normal) B6E13Sn cr/A-Ocgr1cr/J mice (The Jackson Laboratory, Bar Harbor, ME) at 6–8 weeks of age using a collagenase isolation method (16). Pascalic acid inflations were performed with Hanks buffered salt solution (unless otherwise specified), cell culture reagents were purchased from Invitrogen containing 1.6 mg/ml Collagenase P (Roche Applied Science), 4 \( \mu \)g/ml DNase I (Sigma), 9.2 mmol/liter HEPES, 100 units/ml of penicillin, and 100 \( \mu \)g/ml of streptomycin. Islets were purified by centrifugation with a Histopaque gradient (17). Hand-picked islets were placed in Costar ultralow attachment plates (Corning, NY). MIN6 cells were established from \( \beta \)-cell adenomas derived from transgenic mice harboring a hybrid rat insulin promoter-simian virus 40 large T-antigen gene construct (18). MIN6 cells were maintained in 25 cm \(^2\) glucose Dulbecco’s modified Eagle’s medium supplemented with Eagle’s minimal essential medium nonessential amino acid supplement, 44 mm sodium bicarbonate, 15 mm HEPES, 10,000 units/ml penicillin plus 100 \( \mu \)g/ml streptomycin, 15% (vol/vol) fetal bovine serum, 25 \( \mu \)g/ml Fungizone (containing 250 \( \mu \)g/ml amphotericin B and 250 \( \mu \)g/ml sodium deoxycholate). MIN6 cells and islets were maintained at 37 °C in 95% air, 5% CO\(_2\). Experiments were performed when cells were ~70% confluent (passages 20–30) and after islets were precultured for 2 days in 5.6 mm glucose Dulbecco’s modified Eagle’s medium supplemented with 5% heat-inactivated fetal bovine serum.

**Measurement of Butyric acid tolerance**—Proteasome activity was inhibited by lactacystin (purchased from E. J. Corey, Harvard University, Boston, MA). Cells were treated with lactacystin for the times indicated at a final concentration of 10 \( \mu \)M. Lactacystin-treated cultures were then used to assess the effects of lactacystin on glucose-stimulated (pro)insulin secretion.

**Assessment of Cellular Proteinase Activity**—Proteasome activity was assessed in cell lysates using the synthetic peptide substrate Suc-Leu-Leu-Val-Tyr-AMC and release of fluorogenic peptide (19). Untreated control cells underwent routine media change similar to the lactacystin-treated cultures.

**Cellular DNA Quantification and Toxicity Assay**—Total DNA concentrations from crude cell homogenates were determined by fluorometry using bisbenzimide (Hoechst 33258, Molecular Probes, Eugene, OR) as described previously (25). A lactate dehydrogenase-based In Vitro Toxilology Assay kit (Sigma) was used to monitor lactacystin toxicity.

**Confocal Immunofluorescence Microscopy**—Primary antibodies were mouse monoclonal anti-insulin IgG, (Sigma), polyclonal anti-FK2 clone (MBL, Nagoya, Japan), and rabbit anti-GRP78/BiP (StressGen, Victoria, Canada). Secondary antibodies were goat anti-rabbit and anti-mouse secondary fluorescein isothiocyanate conjugates (Sigma). MIN6 cells were seeded at a density of 1 \( \times 10^5 \) cells/chamber in Lab-Tek chamber slides (Fisher Scientific, Pittsburgh, PA). Cells were then preincubated in 25 mm glucose Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum at 37 °C for 4 h. Cells were washed in PBS containing Ca\(^{2+}\) and then fixed for 30 min at room temperature in fresh 3.7% formaldehyde. After fixation, cells were rinsed with PBS, permeabilized with 0.1% Triton X-100 (Sigma) in PBS, and blocked and permeabilized in incubation buffer (0.1% saponin, 1 mm EDTA, 4% goat serum) for 30 min on a gyrating platform. Cells were incubated consecutively with primary and secondary antibodies each for 1 h at room temperature on a gyrating platform. Cells were rinsed five times between and after antibody incubations and mounted with cover glass slides (No. 1, 24 \( \times 50 \) mm; Corning) in glycerol-based SlowFade Light Anti-fade (Molecular Probes). Samples were viewed using an Olympus Fluoview confocal system with Melles Griot Argon (488 nm), Krypton (568 nm), and HeNe (633 nm) lasers with an Olympus BX50 microscope. Negligible staining was detected for each secondary antibody control (negative controls).

**Western Blot Analysis**—MIN6 cells were treated in triplicate to lactacystin (10 \( \mu \)M, 4 h) or tunicamycin (10 \( \mu \)g/ml, 4 h). Equal protein concentrations (15 \( \mu \)g) were size separated by 12% SDS-PAGE, transferred to a nitrocellulose membrane, and probed with polyclonal antibodies to GRP78/BiP, and horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG. Bands were detected with the enhanced chemiluminescence system (Amerham Biosciences). Immunoblots were scanned by optical densitometry to quantify the relative level of protein expression between treatments.

**RNA Extraction and cDNA Synthesis**—Total RNA was extracted using an

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Fig. 1. Pulse-chase designs. A, to assess the effects of proteasome inhibition on glucose-stimulated (pro)insulin synthesis, islets were exposed to lactacystin for 2 h and then \( ^3\text{H} \)-labeled. B, to determine the effects of proteasome inhibition on the fate of newly synthesized (pro)insulin \( ^3\text{H} \)labeled, cells were first \( ^3\text{H} \)labeled and then chased with lactacystin. assay validated in our laboratory following a protocol described previously (21). The antibody used does not distinguish proinsulin and insulin. Thus, the relative concentrations of proinsulin and insulin were not determined in the experimental samples and, hence, the term (pro)insulin is used throughout. Rat insulin was used as a standard. Standards, antibodies, and \( ^3\text{H} \)-labeled insulin were obtained from Linco Research, St. Louis, MO. The inter- and intraassay coefficients of variation were 9 and 3%, respectively. Immunoprecipitable \( ^3\text{H} \)-labeled (pro)insulin (cpm/mg DNA) was measured as described previously (22). In brief, 10 \( \mu \)l of the sonicate or cultured medium (maximum immunoreactive (pro)insulin content 50 ng) was incubated at room temperature for 1 h in the presence of excess guinea pig anti-rat (pro)insulin antibody (Sigma), total binding capacity 300–400 ng of (pro)insulin (22–24). After incubation, 5 ng of Protein A-Sepharose was added in 100 \( \mu \)l of glycine-BSA buffer to the samples. Samples were mixed for 1 h at room temperature. Immunoprecipitate complexes were pelleted by centrifugation at 8,000 \( \times \) for 1 min and washed in phosphate-buffered saline to remove excess debris. Pellets were dissolved in 0.4 ml of 1.5 mM acetic acid, 2.5 mg/ml BSA and transferred to liquid scintillation vials. Radioactivity was determined in a liquid scintillation counter using 6 ml of Aquasol-2 (universal LSC mixture; Packard, Meriden, CT). Using the above method, the percentage of (pro)insulin, which was quantitatively precipitated and recovered (22–24).

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Effects of Lactacystin on Glucose-stimulated (Pro)insulin Biosynthesis and Secretion—Total intracellular (pro)insulin concentrations and total (pro)insulin secretion were similar in control and lactacystin-treated mouse islets exposed to 5.6 mM glucose (data not shown). At 20 mM glucose, total secreted (pro)insulin was reduced significantly by 51% in response to lactacystin (10 \( \mu M \)) treatment for 4 h (Fig. 3A). Total intracellular (pro)insulin content was similar between lactacystin-treated and untreated control islets (Fig. 3B).

Metabolic labeling studies were performed to assess the effects of lactacystin on glucose-stimulated (pro)insulin biosynthesis. (Pro)insulin synthesis was determined after 2 h of exposure to glucose (5.6 or 20 mM) in the absence or presence of lactacystin (10 \( \mu M \), Fig. 3C). The incorporation of \(^{3}H\)tyrosine into (pro)insulin during the 20-min labeling period was taken as the rate of (pro)insulin synthesis (25, 58). As expected, for control islets the rate of (pro)insulin synthesis increased significantly in response to high glucose (20 mM) (29–31). In 5.6 mM glucose, lactacystin did not significantly alter (pro)insulin synthesis. However, (pro)insulin synthesis decreased significantly after 2 h of exposure to lactacystin in high glucose (20 mM).

To assess the effects of lactacystin on the fate of newly synthesized \(^{3}H\)tyrosine-labeled (pro)insulin, islets were first \(^{3}H\)tyrosine-labeled (prior to lactacystin treatment) and then chased in 20 mM glucose for 4 h. The quantity of \(^{3}H\)tyrosine-labeled (pro)insulin released was similar between lactacystin-treated and untreated control islets. However, to determine the proportion of newly synthesized (pro)insulin contributing to the total secreted (pro)insulin pool (Fig. 3D), the specific radioactivities were determined by dividing the secreted \(^{3}H\)tyrosine-labeled (pro)insulin by the total immunoreactive (pro)insulin (non-labeled plus labeled (pro)insulin). The specific radioactivities of \(^{3}H\)tyrosine-labeled (pro)insulin in the cultured medium of control islets treated with 20 mM glucose were 0.25 ± 0.05 and 0.25 ± 0.06 cpm/microunits during the 2- and 4-h chase periods, respectively. For islets treated with lactacystin, the specific radioactivities of \(^{3}H\)tyrosine-labeled (pro)insulin in the cultured medium were 0.29 ± 0.04 and 0.52 ± 0.16 cpm/microunits for the 2- and 4-h chase periods, respectively. Therefore, a significantly higher proportion of newly synthesized (pro)insulin was secreted from lactacystin-treated islets after 4 h of lactacystin treatment. Similar to islets, (pro)insulin synthesis was significantly decreased, whereas a significantly higher proportion of newly synthesized (pro)insulin was secreted from lactacystin-treated MIN6 cells (data not shown).

Intracellular (Pro)insulin Degradation—The data for secreted and intracellular \(^{3}H\)tyrosine-labeled (pro)insulin were used to calculate the effects of lactacystin on intracellular (pro)insulin degradation in mouse islets and MIN6 cells. Degraded intracellular (pro)insulin was determined as the percentage of \(^{3}H\)tyrosine-labeled (pro)insulin (in cells and the medium) that was not recovered during the chase periods (22, 23, 30). During the 4-h chase period, control islets degraded 25 and 8% of \(^{3}H\)tyrosine-labeled (pro)insulin when exposed to 5.6 and 20 mM glucose, respectively. The percentages of \(^{3}H\)tyrosine-labeled (pro)insulin degraded increased to 32 and 24% (\( p < 0.05 \)) in response to lactacystin in 5.6 and 20 mM glucose, respectively.

Extracellular degradation of \(^{3}H\)tyrosine-labeled (pro)insulin (in the medium) might affect the interpretation of the pulse-chase data and assessment of (pro)insulin turnover. However, if extracellular (pro)insulin degradation occurred to a significant extent, control and lactacystin treatments would have been affected equally because the two treatments were incubated under similar conditions. Therefore, the significant differences observed between control and lactacystin-treated cul-

**RESULTS**

Effects of Lactacystin on Inlet Proteasome Activity—Islets were exposed to 5.6 mM glucose ± lactacystin (10 \( \mu M \)) for up to 4 h to measure the temporal effects of lactacystin on proteasome activity. Lactacystin treatment significantly reduced proteasome activity in islets by 91% after 1 h and by 99% after 4 h of exposure (Fig. 2).

**FIG. 2. Effects of lactacystin on proteasome activity in islets.** To examine the effects of proteasome inhibitor lactacystin on islet proteasome activity, 100 islets in triplicate were exposed to 5.6 mM glucose ± lactacystin (10 \( \mu M \)) for up to 4 h (white bar, control; black bars, lactacystin). Proteasome activity was measured as described under "Experimental Procedures."

with TRIzol® reagent (Invitrogen) from MIN6 cells treated similarly as for Western blot analysis. After quantification by spectrophotometry, equal quantities of RNA/treatment were reverse-transcribed to cDNA using a GeneAmp PCR System 2400 thermocycler (Applied Biosystems, Foster City, CA) in a final reaction volume of 50 \( \mu l \) containing SYBR Green PCR Master Mix (Applied Biosystems), 0.5 \( \mu l \) of (each) primer, and 5 \( \mu l \) of cDNA template. The primers used to detect 18 S RNA (137 bp) were forward-(5'-CAAAGCCCTCG-3') and reverse-(5'-CACATCCG-3').

The data for secreted (pro)insulin (non-labeled plus labeled (pro)insulin) was used to calculate the effects of lactacystin on intracellular (pro)insulin degradation in mouse islets and MIN6 cells. Degraded intracellular (pro)insulin was determined as the percentage of \(^{3}H\)tyrosine-labeled (pro)insulin (in cells and the medium) that was not recovered during the chase periods (22, 23, 30). During the 4-h chase period, control islets degraded 25 and 8% of \(^{3}H\)tyrosine-labeled (pro)insulin when exposed to 5.6 and 20 mM glucose, respectively. The percentages of \(^{3}H\)tyrosine-labeled (pro)insulin degraded increased to 32 and 24% \( (p < 0.05) \) in response to lactacystin in 5.6 and 20 mM glucose, respectively.

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Fig. 3. Effects of lactacystin on glucose-stimulated (pro)insulin biosynthesis and secretion. Total immunoreactive (pro)insulin concentrations (secreted, A; intracellular, B) were measured in response to 20 mM glucose in the absence or presence of lactacystin (10 μM) for 2 or 4 h. Glucose-stimulated (pro)insulin synthesis (C) was determined by the incorporation of [3H]tyrosine after 2 h of exposure to glucose (5.6 or 20 mM) in the absence or presence of lactacystin (10 μM). To determine the fate of newly synthesized ([3H]tyrosine-labeled) (pro)insulin, islets were first [3H]tyrosine-labeled and then chased in glucose (5.6 or 20 mM) in the absence or presence of lactacystin (10 μM) for up to 4 h. The specific radioactivities of [3H]tyrosine-labeled (pro)insulin in the culture medium are shown (D). Data are expressed as the mean of triplicates ± S.E. and represent microunits/μg DNA or cpmp/μg DNA (white bars, control; black bars, lactacystin). *, p < 0.05 versus control.

Analysis of ER Chaperones and Expression of the Stress-inducible Transcription Factor CHOP/GADD153—To determine whether proteasome inhibition elicits ER stress, the mRNA expression of ER chaperones GRP78/BiP (classic marker of ER stress), GRP94, and PDI were examined by real-time qRT-PCR in MIN6 cells. Realtime qRT-PCR analysis demonstrated that lactacystin (10 μM, 4 h) significantly up-regulated GRP78/BiP, GRP94, and PDI mRNA expression (Fig. 5A). Quantitation of GRP78/BiP protein by Western blot analysis demonstrated GRP78/BiP protein concentrations were similar in response to 4 h of lactacystin treatment (10 μM, Fig. 5B). To further assess the effects of lactacystin on the ER chaperone GRP78/BiP, confocal immunofluorescence microscopy was performed. Representative images from one of five independent experiments performed in triplicate are shown (Fig. 5C). In control  β-cells, GRP78/BiP revealed a classic perinuclear ER-like staining (Fig. 5C, left panel). In contrast, GRP78/BiP in lactacystin and tunicamycin-treated β-cells was redistributed to juxtanuclear regions consistent with the presence of aggregates or potential "quality control compartments" derived from the ER (Fig. 5C, center and right panels) (32).

The gene encoding C/EBP-homologous protein (CHOP), also known as growth arrest and DNA damage-inducible gene 153 (GADD153), is a stress-inducible transcription factor that is activated by agents that adversely affect the function of the ER (33). Expression of CHOP/GADD153 mRNA was detected by RT-PCR after exposure of β-cells to lactacystin (10 μM, 4 h) and tunicamycin (10 μg/ml), but not in control β-cells (Fig. 5D).
The Proteasome and β-Cell Function

Detection of Polyubiquitinated Proteins—MIN6 cells treated with lactacystin (10 μM) for 4 h were assayed for polyubiquitinated proteins. A significantly increased polyubiquitinated immunoreactivity that comprised a wide molecular mass range relative to equivalent protein concentrations of control cells was detected after 4 h of exposure to lactacystin (Fig. 6). A polyubiquitinated protein or protein complex (~22 kDa) was detected in control and tunicamycin-treated β-cells. The band intensity of this unknown protein decreased in response to lactacystin.

Confocal immunofluorescence microscopy was performed to further assess the observed increase in polyubiquitinated proteins in response to lactacystin and tunicamycin. In agreement with the immunoblot data, polyubiquitinated proteins appeared to increase in quantity and to accumulate in the cytosol relative to the nucleus after lactacystin treatment (Fig. 6C, center panel). In the absence of lactacystin treatment, polyubiquitinated proteins were apparent in both nuclei and cytosol, these likely representing proteins targeted for normal turnover (Fig. 6C, left panel). The staining of polyubiquitinated proteins in tunicamycin-treated β-cells (Fig. 6C, right panel) was similar to untreated control β-cells.

**Fig. 4. Effects of lactacystin on (pro)insulin expression.** A, an ethidium bromide-stained agarose gel showing the correctly sized RT-PCR products for insulin1/2 (143/149 bp) and GAPDH (225 bp, constitutive control) obtained from lactacystin-treated MIN6 cells. B, steady-state mRNA concentrations of insulin1/2/GAPDH as determined by qRT-PCR are shown. (white bar, control; black bar, lactacystin). Representative data from one of three independent experiments performed in triplicate are shown; means ± S.E. C, confocal immunofluorescence and phase contrast images of MIN6 cells immunostained for (pro)insulin (yellow). LC, lactacystin; Tm, tunicamycin. Representative images from one of five independent experiments performed in triplicate are shown.

**DISCUSSION**

The present data demonstrate in pancreatic β-cells that proteasome inhibition activated an unfolded protein response (UPR), which significantly altered glucose-stimulated (pro)insulin biosynthesis and its compartmental distribution. Specifically, the rate of (pro)insulin biosynthesis was reduced in lactacystin-treated islets and MIN6 cells, although steady-state insulin mRNA concentrations were unaffected, suggestive of translation attenuation. These observations together with the increased expression of ER chaperones and induction of the stress-inducible transcription factor CHOP/GADD153 are consistent with the activation of a UPR. The reduction in (pro)insulin secretion by proteasome inhibition was associated with a significant decrease in total glucose-stimulated (pro)insulin secretion from both islets and MIN6 cells. Specifically, in high glucose (20 mM), a decrease in total glucose-stimulated (pro)insulin secretion by islets was evident after 4 h of exposure to lactacystin. However, basal (pro)insulin release at 2.8 (data not shown) and 5.6 mM glucose was similar between control and lactacystin-treated β-cells. These data are in agreement with a recent report describing unpublished data, in which lactacystin affected neither the conversion of endogenous rat proinsulin to insulin nor basal insulin secretion (15).

The observed release of a greater proportion of newly synthesized (pro)insulin by proteasome-inhibited cells was unexpected. Although the underlying mechanism is presently unclear, several possibilities may be considered. Lactacystin may inhibit primarily the release of stored (pro)insulin with little effect on the release of newly synthesized (pro)insulin. In other words, release of newly synthesized (pro)insulin is maintained at a constant rate (similar rate relative to untreated control), whereas only the secretion of stored (pro)insulin (unlabeled or preformed pool) decreases. In this case, an increased capacity of ER chaperones to facilitate protein folding during the UPR likely contributes to an increased efficiency by which newly synthesized (pro)insulin passes through the secretory pathway. Glucose itself increases the preferential release of newly synthesized (pro)insulin (65), and proteasome inhibition may potentiate this glucose effect. However, the underlying mechanism for preferential release of newly synthesized (pro)insulin is not known. Previous studies have shown that an integrated regulation of (pro)insulin synthesis, degradation, and secretion appears to adapt in a coordinated manner to changes in the extracellular environment (23). Similarly, a functional coordination between the UPR and ERAD pathways has been demonstrated in yeast (7, 8). We question the extent to which glucose regulation of proteasome activity may contribute to the integrated coordination among (pro)insulin synthesis, degradation, and secretion. The differential extent to which (pro)insulin secretion was inhibited by lactacystin at high (20
mM) relative to the lower 5.6-mM glucose concentration suggests that proteasomal activity may be glucose-sensitive. However, proteasome activities were similar between islets incubated for 4 h in 5.6 versus 20 mM glucose. Clearly, additional studies are necessary to further examine this potential interaction and its effects on glucose homeostasis in vivo, especially because a role of ER stress in the pathophysiology of common forms of diabetes appears to be emerging (34).

Previous reports (22–24, 35–39) have established that intracellular insulin degradation contributes to the maintenance of insulin content in β-cells. Particularly, the intracellular degradation of stored insulin was postulated (23, 24, 39) to occur by the regulated existence of two granule pools (pool 1 for release and pool 2 for degradation). Subsequent studies that followed the time course of lysosomal alterations after rapid changes in glucose concentration indicated that lysosomes are involved mainly in the long-term adaptation of the β-cell to varying functional demands (37). These studies focused on the regulation of intracellular insulin degradation at the distal portion of the secretory pathway. Although lysosomal proteolysis has classically been considered the major site for protein degradation, the potential influence of regulated degradation or processing of proinsulin and its intermediates occurring early in the secretory pathway (e.g. ERAD by the proteasome) cannot be excluded.

Misfolded proteins are dislocated out of the ER lumen and subsequently degraded by the proteasome, which resides primarily in the cytosol (40–46). In view of this current model of the ERAD degradation pathway (40–46) and the “fidelity of protein biogenesis” (2), in which upwards of 30% of newly synthesized proteins depend on the proteasome for their rapid removal, and the fact that (pro)insulin is a major ER client protein in pancreatic β-cells, a prevesicular (pro)insulin degradation pool might exist that should be detectable only in cells limited by an inactive proteasome. With proteasome inhibition, we predict the gradual accumulation of proteins in the ER lumen, which upon reaching a critical “set-point” concentration, activates the UPR pathway, resulting in translational attenuation and leading to an initial increase in the release of newly synthesized (pro)insulin (both native and potentially defective molecules). Hence, the proteasome might function primarily as an immediate rather than long-term protein degradation system (37), which likely influences the quality and quantity of (pro)insulin stored or secreted. However, in the present studies, the quantity of newly synthesized (pro)insulin recovered in response to proteasome inhibition is not entirely consistent with this view. Specifically, lactacystin did not prevent, but enhanced, the intracellular degradation of (pro)insulin in islets. Thus, the proteasome likely does not play a direct role in the intracellular degradation of wild-type (pro)insulin in pancreatic β-cells. It is unclear how lactacystin treatment increased intracellular (pro)insulin degradation. As a possibility, proteolytic enzymes in the ER lumen may degrade the aggregated (pro)insulin if it is returned there (47–50). Recent data,
however, do support proteasome-mediated degradation of a significant portion of mutant proinsulin 2 in rat islets (15) and a transfected Chinese hamster ovary cell line (51). Proteasome-mediated degradation clearly contributes to quality control in the ER (41–45, 52), and its relevance for pancreatic β-cell function is demonstrated in the Akita mouse model (Ins2Akita), which carries a conformation-altering missense mutation (C96Y) in insulin 2 that disrupts one of the two disulfide bonds between the insulin A and B chains (9, 10). Overall (pro)insulin transport from the ER to the Golgi was found largely blocked, and mutant (pro)insulin, complexed with GRP78/BiP, accumulated in the ER lumen (10). The characteristic diabetic phenotype for this model may reflect increased ER stress, by production of mutant (pro)insulin that has exceeded the capacity of the proteasome. Cells genetically engineered to produce protein aggregates demonstrated that a threshold exists above which the proteasome is overwhelmed (53). As in Akita mouse islets, characteristic GRP78/BiP aggregates were observed in response to proteasome inhibition. Further, expression of the chaperones GRP78/BiP, GRP94, and PDI was up-regulated, and polyubiquitinated proteins comprising a wide molecular mass range accumulated in proteasome-inhibited MIN6 cells. ER resident chaperones such as GRP78/BiP prevent protein aggregation by facilitating folding processes and are thought to contribute to the dislocation of misfolded proteins to the cytosol for degradation (54). Indeed, chaperonin retention in the ER lumen is one mechanism whereby defective proteins are prohibited from proceeding through the secretory pathway (55–57). Extensive retention and the buildup of protein aggregates eventually congests the ER, leading first to translational attenuation and finally to apoptosis when functions of the ER are severely impaired.

Although apoptosis was not apparent after lactacystin treatment in the present studies, CHOP/GADD153 expression was detected. Recently, it was demonstrated that a background mutation in CHOP/GADD153 would ameliorate ER stress-mediated cell death in the Akita mouse (58). The gene encoding CHOP/GADD153 is induced by ER stress through a signaling pathway that involves activation of PERK and phosphorylation of the translation initiation factor eIF2α. Identified as the eIF2α kinase enriched in pancreatic cells (59), PERK is an ER transmembrane protein whose activity is repressed by GRP78/BiP. When misfolded proteins accumulate in the ER, GRP78/BiP dissociates from PERK, resulting in the activation of this kinase, which then phosphorylates eIF2α, a master regulator of translation (60–62). By halting translation initiation and protein synthesis, PERK is thought to relieve ER stress by reducing the generation of proteins in the ER (11, 60–62) and to promote the survival of β-cells, thus contributing to glucose homeostasis. The overall decrease in (pro)insulin synthesis observed in proteasome-inhibited mouse islets and MIN6 cells is consistent with translational attenuation.

In summary, the present data implicate a novel role for the ubiquitin-proteasome system in regulating glucose-stimulated (pro)insulin synthesis and its compartmental distribution in pancreatic β-cells. Proteasome inhibition elicited ER stress in β-cells and induced a UPR, which increased the proportion of newly synthesized (pro)insulin released despite decreased (pro)insulin synthesis. The complexity of the UPR is revealed by transcriptional up-regulation of over 350 genes in yeast (7, 8), the majority of which function in various aspects of the secretory pathway. The characterization of such ER stress-induced genes in β-cells will potentially reveal novel loci that may help define the genetic basis of diabetes. Recently, it was demonstrated that mutant
(proinsulin that cannot be converted is secreted, albeit as a low portion of total (proinsulin, from primary rat β-cells via the regulated pathway (15). The crucial implication of this together with the present findings is that inefficient or faulty recognition and elimination of misfolded proteins, particularly the increased release of proinsulin (or biologically defective (pro)insulin) (15, 51, 63, 64), could contribute to either insulin resistance or β-cell dysfunction, or both.

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