

Endoplasmic Reticulum Oxidoreductin-1 α (Ero1 α) Improves Folding and Secretion of Mutant Proinsulin and Limits Mutant Proinsulin-induced Endoplasmic Reticulum Stress*

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Background: Mutant proinsulin-G(B23)V is defective in formation of native disulfide bonds.

Results: Overexpression of Ero1 α partially rescues secretion of mutant proinsulin-G(B23)V.

Conclusion: Enhanced ER oxidation is of potential benefit to proinsulin folding.

Significance: Diseases involving proinsulin misfolding might be ameliorated by techniques augmenting protein oxidation in the ER of pancreatic β -cells.

Upon chronic up-regulation of proinsulin synthesis, misfolded proinsulin can accumulate in the endoplasmic reticulum (ER) of pancreatic β -cells, promoting ER stress and type 2 diabetes mellitus. In Mutant *Ins*-gene-induced Diabetes of Youth (MIDY), misfolded mutant proinsulin impairs ER exit of co-expressed wild-type proinsulin, limiting insulin production and leading to eventual β -cell death. In this study we have investigated the hypothesis that increased expression of ER oxidoreductin-1 α (Ero1 α), despite its established role in the generation of H₂O₂, might nevertheless be beneficial in limiting proinsulin misfolding and its adverse downstream consequences. Increased Ero1 α expression is effective in promoting wild-type proinsulin export from cells co-expressing misfolded mutant proinsulin. In addition, we find that upon increased Ero1 α expression, some of the MIDY mutants themselves are directly rescued from ER retention. Secretory rescue of proinsulin-G(B23)V is correlated with improved oxidative folding of mutant proinsulin. Indeed, using three different variants of Ero1 α , we find that expression of either wild-type or an Ero1 α variant lacking regulatory disulfides can rescue mutant proinsulin-G(B23)V, in parallel with its ability to provide an oxidizing environment in the ER lumen, whereas beneficial effects were less apparent for a redox-inactive form of Ero1. Increased expression of protein disulfide isomerase antagonizes the rescue provided by oxidatively active Ero1. Importantly, ER stress induced by misfolded proinsulin was limited by increased expression of Ero1 α , suggesting that enhancing the oxidative

folding of proinsulin may be a viable therapeutic strategy in the treatment of type 2 diabetes.

The single-chain insulin precursor, proinsulin, is the principal protein synthesized in pancreatic β -cells and consists sequentially of the insulin B-chain, C-peptide with flanking cleavage sites, and insulin A-chain. Nascent proinsulin must fold properly including the formation of three native disulfide bridges (B7-A7, B19-A20, and A6-A11) for efficient export from the endoplasmic reticulum (ER)⁴ and delivery to secretory granules for processing and exocytosis of mature insulin (1). Despite the central biomedical importance of this process, the specific enzymes involved in the oxidative folding of proinsulin remain poorly understood (2). The present study focuses on the role of one such enzyme, Ero1 α .

Our studies were motivated by the role of proinsulin misfolding in β -cell dysfunction. Under conditions of increased synthesis, proinsulin is susceptible to increased misfolding involving the mispairing of cysteines to form non-native disulfide bonds (3, 4). Accumulation of misfolded proinsulin in the ER triggers a state known as ER stress, leading to activation of unfolded protein response pathways that may culminate in apoptosis (5) with ultimate loss of pancreatic β -cell mass (6) and type 2 diabetes (7). Indeed, misfolded proinsulin is an established cause of autosomal dominant diabetes in the syndrome of Mutant *Ins* gene-induced Diabetes of Youth (MIDY) (8). The mutant proinsulin molecules are retained in the ER due to a defect in their folding (9); moreover, misfolded mutant proinsulin molecules exert a dominant-negative effect on the export of co-expressed wild-type molecules through direct association (8, 10, 11), leading to ER stress and eventual β -cell death (12).

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⁴ The abbreviations used are: ER, endoplasmic reticulum; hPro, human proinsulin; MIDY, Mutant *Ins*-gene induced Diabetes of Youth; PDI, protein disulfide isomerase; Ero1, ER Oxidoreductin-1; RIPA, radioimmune precipitation assay buffer; EV, empty vector.

In recent years increasing attention has been paid to the role of ER oxidoreductin-1 (Ero1) in promoting the oxidative folding of proinsulin in the ER (13, 14). Mammals express two isoforms of Ero1 (α and β); both are ER luminal flavoproteins that couple reduction of molecular oxygen with the oxidation of ER oxidoreductases such as protein disulfide isomerase (PDI) (15). In turn, the ER oxidoreductases can shuttle disulfide bonds to substrates to catalyze the folding of newly synthesized secretory proteins (16, 17), such as proinsulin (2). Among other pathways, Ero1 is the best-known source of disulfide bonds in the ER lumen (18). Because Ero1 deficiency impairs proinsulin maturation and predisposes to insulin-deficient diabetes (13), it occurred to us that improved proinsulin oxidative folding may provide a novel approach to ameliorating insulin production, especially under states of ER stress in which misfolded proinsulin may block export of bystander proinsulin molecules (19). Propelled by the hypothesis that improvement of proinsulin folding kinetics may overcome ER retention of both mutant and WT molecules, in this study we have examined the effect(s) of increased Ero1 α expression on misfolded proinsulin in the ER. The results suggest that manipulating the β -cell ER proteome may provide a therapeutic window through which proinsulin misfolding may be ameliorated.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—293T cells were cultured in DMEM plus 10% fetal bovine serum and penicillin/streptomycin (100 units/ml; 100 μ g/ml). INS1E cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 1 mM pyruvate, 10 mM HEPES, penicillin/streptomycin (as above), and 50 μ M 2-mercaptoethanol. Flp-In T-Rex 293 cells were stably transfected with empty vector, Ero1 α -WT, or Ero1 α -Active as previously described (20) and maintained in MEM (Sigma, M4526) supplemented with 10% fetal bovine serum, penicillin/streptomycin (as above), 1 \times GlutaMAX (Invitrogen), 7.5 μ g/ml blasticidin, and 50 μ g/ml hygromycin. For induction of gene expression, cells were incubated in complete media containing 1 μ g/ml doxycycline for 24 h.

Proinsulin variants were expressed in pcDNA3.1 (Invitrogen) or pTarget (Promega), Ero1 α variants were expressed in pcDNA5/FRT/TO (Invitrogen), roGFP1-iE_{ER} was expressed in pcDNA3.1, and PDI-FLAG was expressed in pcDNA3.1/V5-His TOPO TA. All plasmids have been previously described except Ero1 α -C94A, -C99A, -C104A, -C131A, -C394A, and -C397A ("Ero1 α -Hex"), which were generated with the QuikChange site-directed mutagenesis kit (Agilent). Plasmids were transfected using Lipofectamine 2000 (Invitrogen) for 293T cells or Metafectene Pro (Biontex) for INS1E and Flp-In T-Rex 293 cells. Total plasmid DNA amount was held constant within each experiment by inclusion of empty vector.

Proinsulin Measurements and Western Blotting—For secretion experiments, 24–48 h post-transfection culture medium was changed and collected overnight. Cells were lysed in RIPA buffer (0.1 M NaCl, 0.2% deoxycholate, 25 mM Tris, pH 7.4, 1% Triton X-100, 0.1% SDS, 10 mM EDTA, pH 8.0, and a proteinase inhibitor mixture). Proinsulin was measured by rat insulin radioimmunoassay (RIA, Millipore) that recognizes insulin and proinsulins of multiple species or human proinsulin-spe-

cific RIA (Millipore) normalized to total cell protein measured by BCA assay (Pierce). For immunoblotting, proteins (10 μ g/lane) were resolved by SDS-PAGE on 4–12% acrylamide gradient gels (NuPAGE), electrotransferred to nitrocellulose, and immunoblotted with either anti-Ero1 α (Santa Cruz), anti-Myc (Immunology Consultant Laboratories), or anti- α -tubulin as a loading control (Sigma). Horseradish peroxidase-conjugated secondary antibodies were from Jackson ImmunoResearch, with proteins visualized by ECL (Millipore).

Metabolic Labeling—At 48 h post-transfection, cells were starved in DMEM lacking cysteine and methionine for 30 min. After pulse-labeling with ³⁵S-labeled cysteine and methionine as indicated, cells were lysed in RIPA buffer containing 2 mM *N*-ethylmaleimide and immunoprecipitated with anti-Myc or anti-insulin antibodies and protein A-agarose. Immunoprecipitates were separated by reducing or non-reducing Tris-Tricine-urea-SDS-PAGE and analyzed by autoradiography as previously described (21). For both immunoblotting and metabolic labeling, gel bands were quantified using ImageQuant software.

ER Oxidation—Oxidation of the ER in Flp-In TRex 293 cells using the HyPer_{ER} sensor and in INS1E cells using Grx1-roGFP1-iE_{ER} was measured as previously described (22, 23). Briefly, Flp-In TRex 293 cells were stably transfected with plasmid expressing the HyPer_{ER} sensor (24) as previously described (23). After induction of expression of Ero1 α variants with 1 μ g/ml doxycycline for 24 h, fluorescence spectra were obtained at steady state or in the presence of 10 mM DTT or 100 μ M H₂O₂, with excitation ranging from 410 to 510 nm and emission at 535 nm. The ratios of fluorescence emission intensities upon excitation at 500 nm and at 420 nm from independent experiments were logarithmically transformed and fitted to a linear model using a batch-specific offset value with calculation as previously described (23). INS1E cells were co-transfected with Ero1 α variant or empty vector and Grx1-roGFP1-iE_{ER}, washed, and incubated in PBS containing 20 mM *N*-ethylmaleimide on ice for 20 min and lysed on ice for 1 h in 100 mM NaPO₄, pH 8, containing 1% Triton X-100 and 200 μ M phenylmethylsulfonyl fluoride. After removal of insoluble cell debris by centrifugation, the supernatant was immunoprecipitated using sulfon-N-hydroxysuccinimide-activated agarose (Thermo Scientific) decorated with anti-GFP. Samples were separated by non-reducing SDS-PAGE and immunoblotted using anti-HA antibodies. Blots were analyzed densitometrically to determine the percentage of oxidized sensor (22).

BiP-luciferase Measurement—24 h post-transfection with proinsulin, Ero1 α , and BiP promoter-driven luciferase expression plasmids, cells were split into 2 separate wells. One well was lysed in Passive Lysis Buffer (Promega), and luciferase was measured using the Dual-Light kit (Promega); the other was lysed in Buffer RLT and homogenized using QIAshredder (Qiagen). RNA was isolated using RNeasy (Qiagen); cDNA was generated using the Superscript III First Strand Synthesis kit (Invitrogen). RT-quantitative PCR was performed using Power SYBR Green (Invitrogen) with human proinsulin-specific primers: 5'-CGCAGCCTTTGT-GAACCAAC-3' (forward) and 5'-TGGGTGTGTAGAAG-AAGCCTC-3' (reverse). Luciferase activity was normalized directly to human proinsulin mRNA levels.

Ero1 Promotes Secretion Rescue of Misfolded Proinsulin

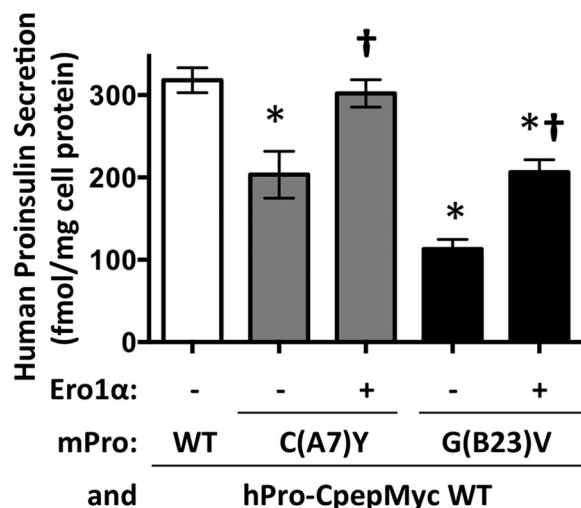


FIGURE 1. Ero1 α rescues wild-type proinsulin in the presence of MIDY mutants. INS1E cells were triple-transfected with plasmids expressing WT hPro-CpepMyc, the indicated mouse proinsulin (*mPro*) mutants, plus Ero1 α or empty vector. At 48 h after transfection, the media were changed for a further overnight collection. Cells were lysed in RIPA buffer, and human proinsulin secretion was measured by human proinsulin-specific RIA normalized to total cellular protein content. Data represent the mean \pm S.E. of three independent transfections. *, $p < 0.05$ versus cells co-expressing WT mouse proinsulin; †, $p < 0.05$ versus cells untransfected with Ero1 α .

Statistical Analysis—Statistical analyses were conducted using GraphPad Prism software. Data are presented as mean \pm S.E. unless otherwise noted. Two-tailed Student's *t* test was used to assess statistical significance, with a threshold for significance of $p < 0.05$. For analysis of HyPer_{ER} data, linear regression, 95% confidence intervals, and *p* values were all calculated using Microsoft Excel.

RESULTS

Ero1 Rescues Wild-type Proinsulin in the Presence of MIDY Mutants—MIDY mutations cause proinsulin to act as a dominant-negative mutant that inhibits WT insulin production (6, 9–11), an effect that may be reversed upon increased expression of Ero1 β and even more by Ero1 α (19). To examine more closely the effect of increased Ero1 expression on this dominant-negative behavior, we co-transfected the INS1E β -cell line with wild-type human proinsulin tagged with a myc-epitope (hPro-CpepMyc) plus either WT or mutant mouse proinsulin. As previously reported (8), mouse mutant proinsulins C(A7)Y or G(B23)V each impaired secretion of co-expressed human WT proinsulin, as measured by human proinsulin-specific radioimmunoassay (Fig. 1). However, co-transfection of Ero1 α in β -cells rescued secretion of WT proinsulin in the presence of mutant proinsulins C(A7)Y or G(B23)V (Fig. 1).

Ero1 Directly Rescues a Selective Subset of MIDY Mutants, with Effects That Are Additive to Secretory Rescue by Wild-type Proinsulin—We wished to determine if prevention of dominant-negative behavior involves primarily Ero1 activity on WT proinsulin or if it might also be attributed to direct effects on mutant proinsulin molecules. With this in mind, we examined the effect of increased Ero1 α expression on the secretion of a variety of MIDY mutant proinsulins expressed in 293T cells in which WT proinsulin is not co-expressed. Remarkably, several proinsulin mutants including G(B8)S and G(B23)V were res-

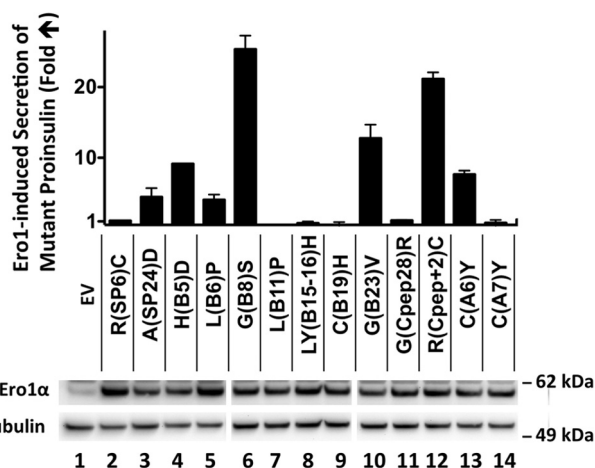


FIGURE 2. Ero1 α rescues secretion of MIDY mutants. 293T cells were transiently co-transfected with plasmids expressing the indicated human proinsulin mutants and Ero1 α or EV. At 24 h post-transfection media were collected overnight, and the cells lysed in RIPA buffer. *Upper panel*, proinsulin secretion was measured by human proinsulin-specific RIA, normalized to total cellular protein content. Data represent the mean \pm range of two independent transfections and are expressed as -fold increase of secretion from cells co-transfected with Ero1 α over secretion from cells co-transfected with EV. *Lower panels*, immunoblots (WB) of cell lysates using anti-Ero1 α antibodies show the level of total Ero1 α expression after Ero1 transfection (*lanes 2–14*) compared with EV (*lane 1* shows the cellular level found in all EV transfections), with α -tubulin used as a loading control. Noncontiguous lanes from the same gel are shown (*white line separators*).

cued upon increased expression of Ero1 α , whereas others including L(B11)P and C(A7)Y could not be rescued (Fig. 2 *lanes 6 and 10 versus lanes 7 and 14*). When examining the different transiently transfected samples, it was clear that the greatest magnitude of secretion rescue did not correlate with samples bearing the highest levels of Ero1 α protein; rather, these results suggest that increased Ero1 α can augment secretion of a subset of MIDY mutants in a manner that depends primarily upon the precise mutation perturbing proinsulin folding.

Wild-type proinsulin can dimerize with mutant proinsulin (25), and wild-type proinsulin can also augment secretion of a similar subset of misfolded MIDY mutants (26). We wished to pursue whether Ero1-mediated secretion rescue phenocopied the effects of WT proinsulin; with this in mind, we examined the effects of these treatments individually, or together on mutant proinsulin secretion. In the presence of WT proinsulin, we observed a modest (~ 3 -fold) increase in secretion of the MIDY mutant proinsulin-G(B23)V (Fig. 3A, *second bar*). Upon increased Ero1 α expression, we observed a much greater increase in proinsulin-G(B23)V secretion (Fig. 3A, *third bar*). When the two treatments were combined, the resulting increase in proinsulin-G(B23)V secretion appeared additive (*fourth bar*). Thus, although secretory rescue may be achieved for the same mutants by both methods, the data imply that Ero1 and WT proinsulin may rescue misfolded proinsulin by distinct mechanisms.

Ero1 α Enhances Folding of Mutant Proinsulin-G(B23)—To examine how increased expression of Ero1 impacts on proinsulin-G(B23)V folding, we co-transfected 293T cells with hProG(B23)V-CpepMyc plus Ero1 α or empty vector and metabolically labeled newly synthesized proteins with 35 S-labeled

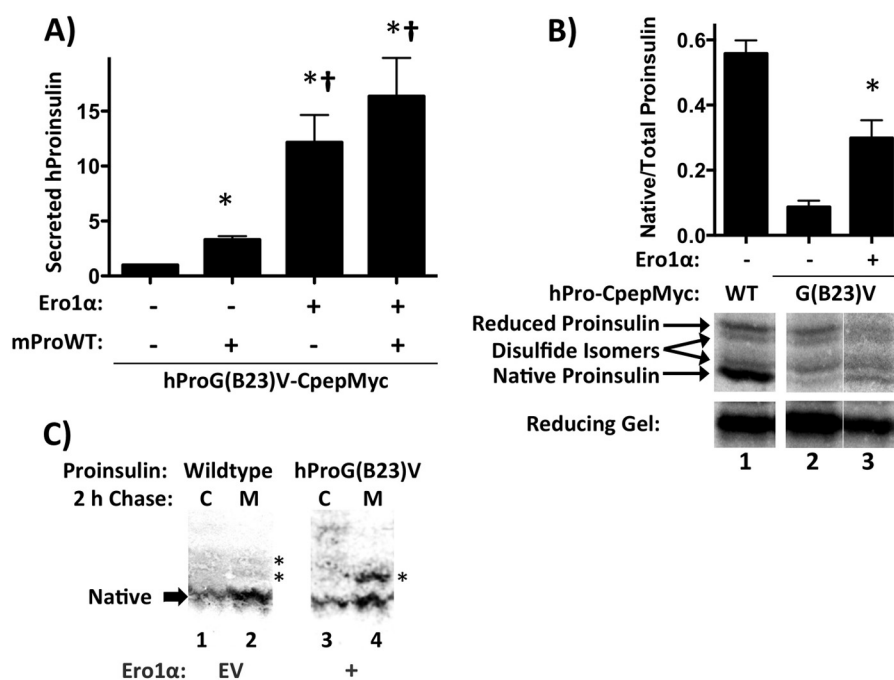


FIGURE 3. Ero1 α enhances folding and secretion of the proinsulin-G(B23)V mutant. *A*, 293T cells were triple-co-transfected with hProG(B23)V-CpepMyc \pm wild-type mouse proinsulin (*mProWT*) \pm Ero1 α . At 24 h post-transfection, cell culture media were collected overnight, and cells were lysed in RIPA buffer. *A*, human proinsulin (hPro) secretion was measured by human-specific RIA normalized to total cellular protein content. Data represent the mean \pm S.E. of three independent transfections, expressed as -fold increase in secretion compared with cells transfected with hProG(B23)V-CpepMyc alone. *, $p < 0.05$ versus the first bar; †, $p < 0.05$ versus the second bar. Between the third and fourth bars, $p = 0.08$. *B*, 293T cells co-transfected with WT or G(B23)V hPro-CpepMyc \pm Ero1 α as indicated were pulse-labeled with ^{35}S -labeled amino acids for 30 min. After washing cells in ice-cold PBS containing 20 mM *N*-ethylmaleimide for 5 min, cells were lysed in RIPA containing 2 mM *N*-ethylmaleimide. After immunoprecipitation with anti-Myc antibodies, samples were analyzed by reducing or nonreducing Tris-Tricine-urea-SDS-PAGE and autoradiography. The nonreduced lane 3 is a longer exposure of the sample to allow direct examination of band intensities relative to lane 2. Data represent the mean \pm S.E. of densitometric quantification of native proinsulin band intensity over total proinsulin intensity. $n = 4$; *, $p < 0.05$ versus cells untransfected with Ero1 α . Noncontiguous lanes from the same gel were spliced together (white line separators). *C*, 293T cells co-transfected with WT or mutant proinsulin, as shown plus EV or Ero1 α and pulse-labeled as in *B*, were chased for 2 h before cell lysis. Both cell lysates (C) and media (M) were analyzed by immunoprecipitation, nonreducing Tris-Tricine-urea-SDS-PAGE, and autoradiography. Noncontiguous lanes from the same gel were spliced together (white space separator, with increased exposure of the hProG(B23)V sample to detect minor bands in the cell lysate). Asterisks denote non-native proinsulin disulfide isomers of unknown identity collected in the medium, one of which is increased from cells expressing hProG(B23)V. Increased secretion of native proinsulin isomer from cells co-expressing hProG(B23)V plus Ero1 α was observed in each of four independent experiments.

amino acids. Immunoprecipitated proinsulin was then analyzed by nonreducing Tris-Tricine-Urea-SDS-PAGE. Proinsulin-WT can be resolved into differentially migrating bands (Fig. 3*B*, lower panel), the fastest of which comprises the majority and represents native proinsulin; the slowest represents fully reduced proinsulin, and intermediate bands represent incompletely or improperly folded proinsulin disulfide isomers (3). Under reducing conditions, all oxidized/partially oxidized proinsulin bands migrate as a single reduced band (Fig. 3*B*, bottom panel). Under nonreducing conditions, the fraction of proinsulin-G(B23)V that achieved the native disulfide-bonded form was <10% of all molecules (Fig. 3*B*, lane 2). Importantly, when Ero1 α was co-transfected, the fraction of proinsulin-G(B23)V achieving a native-like mobility was notably increased (Fig. 3*B*, lane 3), although the fraction of non-native isoforms still remained greater than that seen for WT proinsulin (lane 1). Moreover, upon pulse-labeling and a 2-h chase, much of the secreted proinsulin-G(B23)V from cells co-transfected with Ero1 α migrated under nonreducing conditions like WT proinsulin, comparable in mobility to the native-like intracellular species (Fig. 3*C*). This result contrasts with that from control cells co-transfected with empty vector, in which no bands of secreted proinsulin-G(B23)V could be detected (not shown).

In 293T cells, co-transfection with Ero1 α plasmid typically caused the synthesis of labeled proinsulin-G(B23)V to appear diminished (Fig. 3*B*, lane 3, reduced sample); this was less evident in β -cells and was not correlated with increased ER stress (see below) but may reflect competition between CMV promoters from different plasmids. However, decreased synthesis of proinsulin-G(B23)V in 293T cells cannot readily explain its increased secretion from the same cells (Figs. 2 and 3). Rather, secretory rescue by Ero1 α is correlated with increased oxidation of proinsulin-G(B23)V.

Secretion Rescue Activity of Mutant Proinsulin-G(B23)V by Ero1 α Variants \pm PDI—Oxidase activity of Ero1 α involving the communicating Cys-394--Cys-397 and Cys-94--Cys-99 active-site disulfides is negatively regulated by alternative disulfide linkages involving Cys residues at positions 104 and 131 (20, 27). We utilized a plasmid expressing Ero1 α MycHis₆ with the regulatory Cys residues mutated to Ala; this construct (herein called Ero1 α -Active) has been shown to have increased activity compared with Ero1 α -WT (27, 28). We also mutated the four active sites plus the two regulatory Cys residues in a construct called Ero1 α -Hex (Fig. 4*A*) that lacks the ability to generate disulfide bonds. As measured by immunoblotting, unlike WT proinsulin (Fig. 4*B*, lane 1), a negligible amount of proinsulin-

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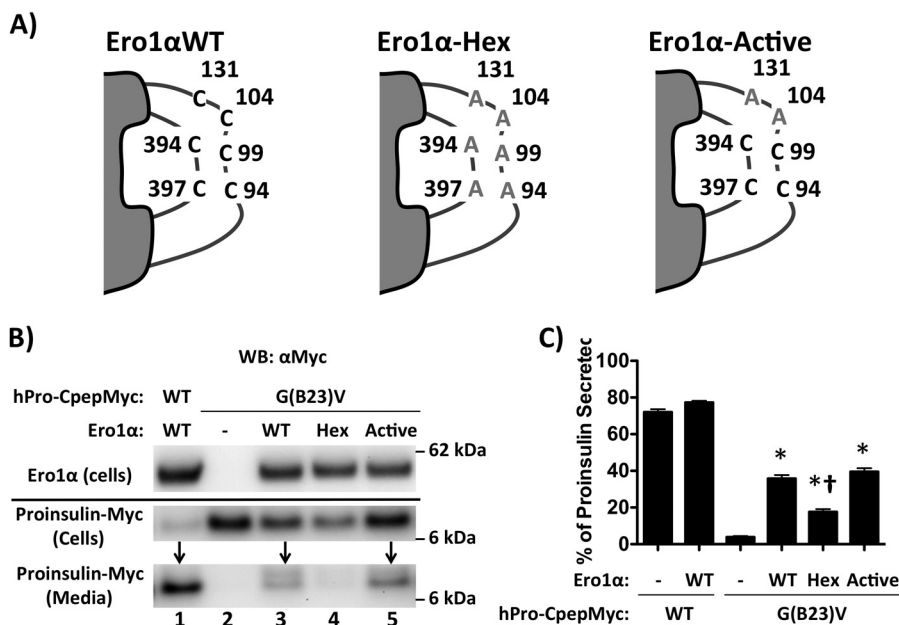


FIGURE 4. The ability of different Ero1 α variants to rescue export of mutant proinsulin-G(B23)V. A, schematic representation of the Ero1 α active sites (C94-C99 and C394-C397) and regulatory cysteines (C104-C131) in WT Ero1 α , Ero1 α -Hex, and Ero1 α -Active. B, 293T cells were co-transfected with the indicated hPro-CpepMyc and Ero1 α MycHis₆ plasmids. At 24 h post-transfection, cell culture media was collected overnight, and cells were lysed in RIPA buffer. Secretion of hPro-CpepMyc and cellular content of hPro-CpepMyc and Ero1 α MycHis₆ were measured by Western blot (WB) analysis using anti-Myc antibodies. Gel loading was normalized to total cellular protein content. C, data represent densitometric quantification of bands from B, expressed as media over total (cell + media) band intensity, $n = 3$. *, $p < 0.05$ versus cells lacking any Ero1 α MycHis₆. †, $p < 0.05$ versus cells co-expressing WT Ero1 α MycHis₆.

G(B23)V could be secreted from cells to medium (Fig. 4B, lane 2). However, co-expression of WT Ero1 α or Ero1 α -Active markedly increased overall proinsulin-G(B23)V secretion (Fig. 4B, lanes 3 and 5). By contrast, enhanced proinsulin-G(B23)V secretion was only minimally affected by co-expression of the Ero1 α -Hex mutant (Fig. 4B, lane 4), although when considered as a fraction of the total (cell + medium) even Ero1 α -Hex promoted a small but statistically significant increase in fractional secretion of mutant proinsulin (Fig. 4C).

To determine if rescue of mutant proinsulin by Ero1 correlated with increased oxidation of the ER lumen, ER redox state and proinsulin secretion were examined in two cell lines; that is, Flp-In T-Rex 293 cells, with inducible expression of either Ero1 α -WT or Ero1 α -Active and transiently transfected INS1E cells. For Flp-In T-Rex 293 cells, ER redox poise upon doxycycline-induced expression of Ero1 variants was measured by the HyPer_{ER} sensor that directly reacts with H₂O₂ and can also be oxidized via ER luminal oxidative folding machinery (23). Induced expression of both Ero1 α -WT and Ero1 α -Active increased the ER luminal oxidation of the HyPer_{ER} reporter; however, the effect for Ero1 α -WT did not achieve statistical significance ($p = 0.058$), whereas significance was achieved for Ero1 α -Active (Fig. 5A). In parallel with the increased oxidative environment of the ER lumen, mutant hProG(B23)V-CpepMyc secretion was augmented (Fig. 5B).

Because transient co-expression of the HyPer_{ER} sensor and Ero1 α variants is unsuitable for subsequent spectral analysis (23), in the INS1E β -cell line we employed the ER-targeted, glutathione-reactive redox-sensitive green fluorescent protein known as Grx1-roGFP1-iE_{ER}, the oxidation state of which can be monitored by mobility shift upon nonreducing SDS-PAGE (22). As expected, ectopic expression of both Ero1 α -WT and

Ero1 α -Active increased oxidation within the ER lumen; however, once again the effect for Ero1 α -WT did not achieve statistical significance ($p = 0.0503$), whereas significance was achieved for Ero1 α -Active (Fig. 5C). Indeed, when examining the results for Ero1 α -WT and Ero1 α -Active in both cell types, the relationship between enhancement of the ER oxidative environment and the extent of proinsulin-G(B23)V secretion rescue suggested more of a threshold effect rather than a direct linear correlation (Fig. 5). Nevertheless, as in Flp-In T-Rex 293 cells, these oxidizing conditions of the ER lumen promoted secretion rescue of mutant proinsulin-G(B23)V (Fig. 5D). By contrast, the inactive Ero1 α -Hex mutant did not stimulate ER luminal oxidation (not shown) and had only minor effects on mutant proinsulin export (Fig. 4).

As part of the canonical ER oxidation pathway, Ero1 is thought to transfer disulfide bonds to ER oxidoreductases such as PDI (29), which can then oxidize substrate proteins. However, recent studies indicate that in pancreatic β -cells (and 293 cells), PDI acts as an "unfoldase" or ER retention factor rather than as an oxidant of proinsulin (2, 30). To determine if PDI enhances or attenuates Ero1 α -mediated secretion rescue of mutant proinsulin, we co-expressed hProG(B23)V-CpepMyc with PDI and Ero1 α . Remarkably, rescue of mutant proinsulin secretion in cells with increased expression of Ero1 α -WT or Ero1 α -Active was significantly impaired by co-expression of PDI (Fig. 6 lanes 3 and 4 and lanes 7 and 8), strongly suggesting that Ero1 α -enhanced proinsulin secretion was not mediated by PDI-catalyzed oxidation. However, co-expression of PDI had no effect in cells expressing the inactive Ero1 α -Hex (Fig. 6, lanes 5 and 6). Thus, the activity of PDI to limit mutant proinsulin export depends on the presence of active site cysteines in Ero1 α .

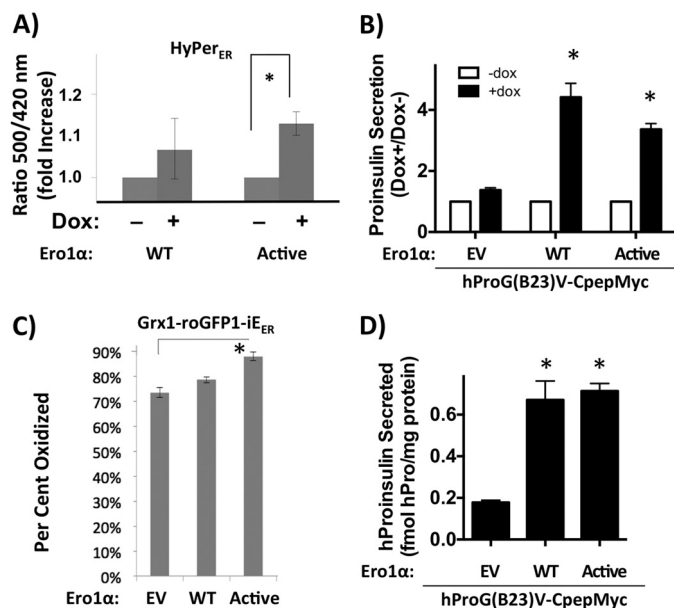


FIGURE 5. Hyperoxidation of the ER lumen promotes export of mutant proinsulin-G(B23)V. A, Flp-In T-Rex-293 cells inducibly expressing Ero1 α MycHis₆-WT or Ero1 α MycHis₆-Active were stably transfected to express the HyPer_{ER} sensor and treated with or without 1 μ g/ml doxycycline (Dox) for 24 h. Fluorescence spectra of HyPer_{ER} sensor in the cells were obtained with appropriate controls (see “Experimental Procedures”). The Dox-induced –fold increase in the 500/420-nm fluorescence ratio along with 95% confidence intervals ($n \geq 3$) is shown; *, $p < 0.05$. B, Flp-In T-Rex-293 cells were transiently transfected with hProG(B23)V-CpepMyc. At 24 h post-transfection, cells were split into separate wells and treated with or without Dox as in panel A. Cell culture media were then collected overnight. The Dox-induced –fold increase of proinsulin secretion (measured by insulin RIA, normalized to total cellular protein content) is shown; *, $p < 0.05$ versus Dox cells. A stable clone of Flp-In T-Rex-293 cells inducibly expressing only EV is also included; panels A and B represent data from three independent experiments. C, INS1E cells transiently expressing the Grx1-roGFP1-iE_{ER} oxidation reporter and either EV or the indicated Ero1 α MycHis₆ variant were analyzed by nonreducing SDS-PAGE and Western blot to determine the percentage of Grx1-roGFP1-iE_{ER} oxidation; * $m p < 0.05$ versus EV. D, INS1E cells transiently co-transfected with hProG(B23)V-CpepMyc and EV or the indicated Ero1 α MycHis₆ variant. At 24 h post-transfection cell culture media were collected overnight, and cells were lysed; proinsulin secretion was measured by human proinsulin-specific RIA normalized to total cellular protein content. The data in panels C and D represent the mean \pm S.E. from 3–4 independent experiments; *, $p < 0.05$ versus EV.

Ero1 α Does Not Augment ER Stress Induced by Proinsulin-G(B23)V—Expression of hyperactive Ero1 α can activate ER stress responses (27), presumably caused by increased generation of H₂O₂ (31). It was, therefore, of interest to know if Ero1-augmented oxidation in cells expressing proinsulin-G(B23)V triggers increased ER stress. To test this in pancreatic β -cells, we co-transfected INS1E cells with a BiP promoter-driven luciferase (BiP-luciferase) reporter as a readout of ER stress signaling and normalized the luciferase signal directly to the mRNA expression of the misfolded substrate driving the ER stress response (32). Consistent with previous reports (8), the G(B23)V mutation triggered a ~2.4-fold increase in BiP-luciferase compared with cells expressing wt proinsulin. However, increased expression of Ero1 α -WT did not augment ER stress signaling by proinsulin-G(B23)V; indeed it resulted in a statistically significant diminution of the G(B23)V-mediated increase in BiP-luciferase (Fig. 7, lane 3). Expression of Ero1 α -Active also tended to inhibit the G(B23)V-

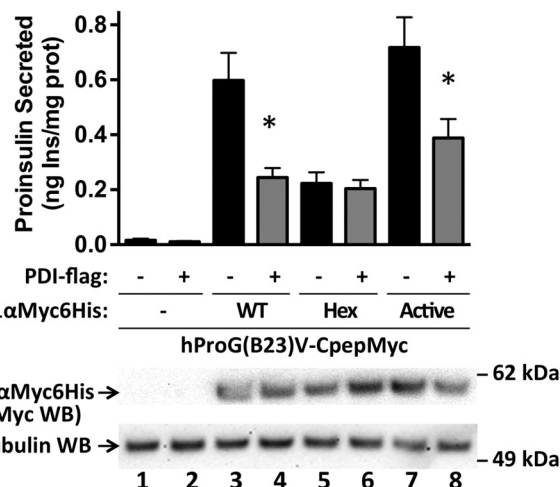


FIGURE 6. PDI antagonizes the ability of Ero1 α to promote proinsulin-G(B23)V secretion. 293T cells were triple-transfected with hProG(B23)V-CpepMyc with the indicated Ero1 α MycHis₆ mutants with or without PDI-FLAG. At 24 h post-transfection, cell culture media were collected overnight, and cells were lysed in RIPA buffer. Proinsulin secretion was detected by insulin RIA normalized to total cellular protein content. Data represent the mean \pm S.E. from four independent experiments. *, $p < 0.05$ versus control cells lacking PDI-FLAG. Ero1 α MycHis₆ was confirmed by anti-Myc immunoblot (WB), with α -tubulin as a gel loading control.

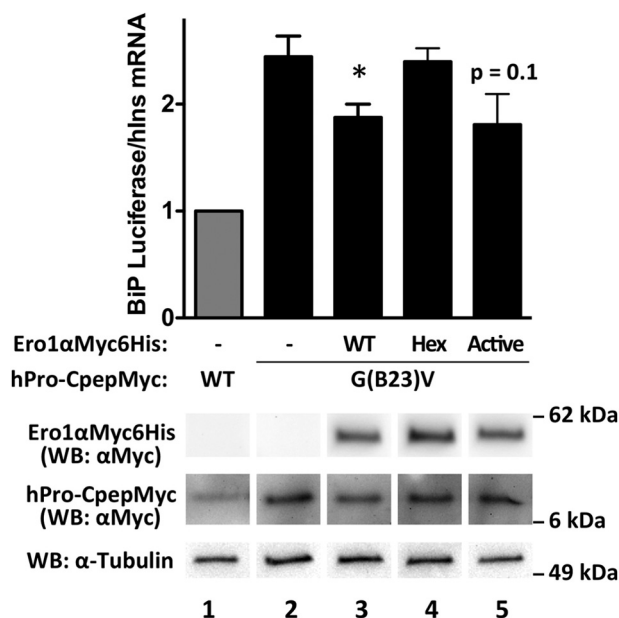


FIGURE 7. Ero1 α limits ER stress activated by proinsulin-G(B23)V. INS1E cells were triple-transfected with plasmids expressing BiP promoter-driven luciferase and the indicated hPro-CpepMyc and Ero1 α MycHis₆ mutants. Transfected cells were split into 3 separate wells, and 48 h after transfection cells were lysed. Lysates were either used to measure luciferase signal by luminometer, human proinsulin mRNA by RT-quantitative PCR, or hPro-CpepMyc and Ero1 α MycHis₆ content by anti-Myc immunoblot (WB) with α -tubulin as a loading control. Data represent the mean \pm S.E. of five independent experiments. *, $p < 0.05$ compared with cells expressing hProG(B23)V-CpepMyc without Ero1 α MycHis₆, expressed as –fold increase of luciferase signal over human insulin mRNA compared with cells expressing WT proinsulin. Noncontiguous lanes from the same gel were spliced together (white line separators).

mediated increase in BiP-luciferase (Fig. 7, lane 5), whereas Ero1 α -Hex did not alleviate the ER stress response induced by mutant proinsulin (lane 4). The data show that an increase of active Ero1 helps to limit ER stress generated by misfolded proinsulin.

DISCUSSION

β -cells are specialized for the manufacture of proinsulin, yet highly up-regulated proinsulin synthesis as occurs in early type 2 diabetes promotes proinsulin misfolding and ER stress (4, 33–37). MIDY mutants offer an opportunity to study the effects of proinsulin misfolding on β -cell dysfunction. These dominant mutants associate with and inhibit intracellular transport of co-expressed wild-type proinsulin (6, 9–11) and can result in eventual ER stress-induced β -cell death (12). An initial report indicated that increased expression of ER oxidoreductin-1 can ameliorate this dominant-negative effect (19). Yet because oxidative stress has been linked to β -cell dysfunction in diabetes (38) and because Ero1 activity includes H_2O_2 generation (presumed to be detrimental to cell health (5, 7, 39)), published studies have attempted to improve β -cell function and viability by decreasing Ero1 levels (13, 14). Although scavenging cellular reactive oxygen species in β -cells may have benefits (40), in fact decreased Ero1 levels diminish β -cell insulin content and sensitize β -cells to ER stress (14). Indeed, mice expressing the MIDY mutant proinsulin-C(A7)Y develop earlier- and more severe insulin-deficient diabetes when even one Ero1 β allele is lacking (13). It has recently been argued that critical re-evaluation is needed of the widely held concept that Ero1-mediated oxidation in the ER contributes to cellular demise (41).

In the current study we have concentrated on proinsulin-G(B23)V as a model of proinsulin misfolding based on *in vitro* folding and structural biology considerations (8, 42). Position B23 contributes to a flexible solvent-exposed β -turn interposed between an α -helix (B9-B19) and β -strand (B24-B28) thought necessary to prime native oxidative maturation, especially the initial C(B19)-C(A20) disulfide pairing (42). The G(B23)V substitution provides a dihedral angle that limits flexibility of the β -turn. Although G(B23)V (and selected other MIDY mutants) does not cause structural perturbation of the native state once achieved (43), this MIDY mutation is expected to impose a critical kinetic barrier to proinsulin foldability (8). Thus, G(B23)V provides an ideal model with which to test possible therapeutic interventions.

The enzymology underlying oxidative folding in the ER lumen defines an important frontier of pharmacology with potential application to diverse diseases of proteotoxicity. Our work started out to confirm that increased expression of Ero1 α attenuates the defect in endogenous insulin production in β -cells exogenously expressing mutant proinsulin-C(A7)Y or G(B23)V (Fig. 1); however, Ero1 α does not at all improve the secretion of proinsulin-C(A7)Y itself (Fig. 2, *lane 14*). Thus in this instance, beneficial effects of Ero1 are likely explained by actions on the co-expressed WT proinsulin partner. Nevertheless, increased expression of Ero1 α improves secretion of a subset of MIDY mutants including proinsulin-G(B23)V even in the absence of any co-expressed wt proinsulin (Fig. 2, *lane 10*). These data strongly suggest a beneficial effect of Ero1 on the mutant proinsulin itself. We recently reported that secretion of proinsulin-G(B23)V is increased in cells co-expressing WT proinsulin (26). Interestingly, many of the same MIDY mutants whose secretion can be enhanced by co-expression with WT proinsulin are among those whose secretion can also be

enhanced by increased expression of Ero1 α . However, Ero1 α exerts a much stronger effect on proinsulin-G(B23)V secretion than does co-expression of WT proinsulin (Fig. 2). In fact, the two treatments together have an additive effect, implying that Ero1 and WT proinsulin may rescue misfolded proinsulin by distinct mechanisms.

An intriguing problem is posed by the mechanism of secretion rescue of misfolded proinsulin by Ero1. In this report we find that increased Ero1 expression enhances the fraction of newly synthesized proinsulin-G(B23)V achieving a native disulfide bonding pattern (Fig. 3B), and this is the form primarily recovered in the rescued secretion (Fig. 3C). Moreover, both in 293 cells and β -cells, increased expression of Ero1 α variants that produce increased oxidation of the ER lumen (Fig. 5, A and C) are accompanied by secretory rescue of misfolded proinsulin (Fig. 5, B and D), whereas Ero1 α -Hex, which lacks the ability to promote disulfide bonds via the canonical mechanism, exhibits markedly decreased proinsulin rescue (Fig. 4, B and C). From these results we hypothesize that rescue of proinsulin misfolding by Ero1 is at least in part attributed to improved kinetics of proinsulin oxidative folding, especially the C(B19)-C(A20) disulfide pairing. However, ER-based oxidation remains poorly understood. The best-studied Ero1 substrate is PDI, and until recently it was assumed that PDI shuttles disulfide bonds to nascent proinsulin. However, new evidence has suggested that PDI actually retards insulin production in β -cells and limits proinsulin intracellular transport (2, 30). Herein we find that co-transfection of PDI attenuates the rescue of misfolded proinsulin provided by Ero1 α -WT or Ero1 α -Active, whereas it had no effect in cells expressing the catalytically inactive Ero1 α -Hex (Fig. 6). These data suggest that PDI either (a) inhibits the enzymatic activity of Ero1 α (which is unlikely), (b) competes with other ER oxidoreductases involved in proinsulin folding, or (c) acts directly on proinsulin in a manner that actually limits proinsulin folding and transport. Indeed, a direct physical interaction between PDI and proinsulin in the ER has been demonstrated (2). Altogether, the current data clearly support the conclusion that oxidative folding of proinsulin, promoted by Ero1, is not mediated by PDI. Nevertheless, our results suggest the possibility, counter to current thinking (40), that augmenting the oxidative capacity of the ER in β -cells may be a reasonable therapeutic strategy for the treatment of proinsulin misfolding that occurs when its synthesis is excessively up-regulated (3, 4), such as in early type 2 diabetes. Accordingly, we found that both Ero1 α -WT and Ero1 α -Active limited the ER stress response to proinsulin-G(B23)V in β -cells, whereas Ero1 α -Hex was without effect (Fig. 7). These data provide support for the notion that Ero1-mediated oxidation of the ER lumen may be beneficial to cells (41), especially in light of recent findings that H_2O_2 generated by Ero1 may be consumed by con-current activity of other gene products (44, 45).

Although promotion of oxidative folding of molecules with kinetic impairment of the proinsulin folding pathway is a critical contribution of Ero1, we note that cells expressing Ero1 α -Hex also showed a modest fractional enhancement of proinsulin secretion (Fig. 4C). Likewise, we did not observe a precise correlation between the fractional increase of ER luminal oxidation and the extent of proinsulin-G(B23)V secretion rescue

(Fig. 5). Together, these data raise the possibility that Ero1 α has additional activities or protein partners that may influence the folding and secretion of proinsulin. Further investigation is needed to explore the magnitude of these effects on secretory protein substrates and to understand the underlying mechanism and significance of Ero1 α oxidative and non-oxidative activities. Ultimately, we believe that ER-targeted therapies, such as that presented as a proof of principle in this report, offer a novel opportunity to intervene in the pathogenesis of β -cell dysfunction in type 2 diabetes mellitus.

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