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Misfolded proinsulin in the endoplasmic reticulum during development of beta cell failure in diabetes

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Short title: Diabetogenic misfolding of proinsulin

Abstract
The endoplasmic reticulum (ER) is broadly distributed throughout the cytoplasm of pancreatic beta cells, and this is where all proinsulin is initially made. Healthy beta cells can synthesize 6000 proinsulin molecules per second. Ordinarily, nascent proinsulin entering the ER rapidly folds via the formation of three evolutionarily conserved disulfide bonds (B7–A7, B19–A20, and A6–A11). A modest amount of proinsulin misfolding, including both intramolecular disulfide mispairing and intermolecular disulfide-linked protein complexes, is a natural by-product of proinsulin biosynthesis, as is the case for many proteins. The steady-state level of misfolded proinsulin — a potential ER stressor — is linked to (1) production rate, (2) ER environment, (3) presence or absence of naturally occurring (mutational) defects in proinsulin, and (4) clearance of misfolded proinsulin molecules. Accumulation of misfolded proinsulin beyond a certain threshold begins to interfere with the normal intracellular transport of bystander proinsulin, leading to diminished insulin production and hyperglycemia, as well as exacerbating ER stress. This is most obvious in mutant INS gene–induced diabetes of youth (MIDY; an autosomal dominant disease) but also likely to occur in type 2 diabetes owing to dysregulation in proinsulin synthesis, ER folding environment, or clearance.

Keywords: secretory protein synthesis; disulfide mispairing; protein aggregation; endoplasmic reticulum (ER) stress; ER-associated degradation; mutant INS gene–induced diabetes of youth (MIDY)

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Introduction

Growing evidence suggests that, during progression of type 2 diabetes, loss of pancreatic beta cell function and eventual loss of beta cell mass is accompanied by endoplasmic reticulum (ER) stress.[1–4] Many factors may contribute to ER stress, but one of the most well recognized of these factors are conditions that promote misfolding of secretory proteins within the ER lumen.[5] The beta cell secretory proteins most cited as prone to misfolding are islet amyloid polypeptide (IAPP) and proinsulin.[6,7] In rodent models of type 2 diabetes, endogenous IAPP does not misfold, and, even in felines, nonhuman primates, and humans, there is little convincing data to establish that nascent pro-IAPP is amyloidogenic during its passage through the ER compartment.[8] By contrast, there is a growing body of evidence to suggest that proinsulin misfolding may occur in the ER, triggering beta cell ER stress. As we discuss below, proinsulin misfolding in the ER is most readily detected by the presence of mispaired disulfide bonds, including both intramolecular and intermolecular disulfide mispairings.

To date, there has been vastly more study of insulin misfolding (a pharmaceutical problem) than proinsulin misfolding (a cell biological problem).[9] As the C-peptide of proinsulin is largely unstructured, the conformational state of proinsulin is dominated by its insulin moiety, comprised of the 30-residue B-chain and the 21 residue A-chain. In the insulin crystal structure, the N-terminal B-chain offers a central α helix and the A-chain provides two additional short α helices. Amino acids L(A16), Y(A19), L(B11), L(B15), and R(B22) have been reported to be the five most importantly positioned residues in stabilizing the folded state of the insulin monomer.[10] Distinct crystallographic structures known as R and T states reflect dynamic differences in amino terminal portion of the B-chain, which can be mostly α-helical (R state) or extended (T state).[11] Like many globular proteins, insulin has a number of buried hydrophobic residues in the native state that may become solvent exposed in incompletely folded states.[12] Such improperly exposed peptide sequences, especially those including the central B-chain helix LVEALYL, are thought to be aggregation prone/amyloidogenic[13] and can associate with neighboring insulin moieties, even when they are in the native state.[14] In the native state, two of the insulin disulfide bonds—C(A6)–C(A11) and C(B19)–C(A20)—are no longer exposed to solvent, and both burying and consumption of the C(A11) thiol are particularly important for the proinsulin folding pathway.[15]

In beta cells, proinsulin synthesis begins as preproinsulin polyribosomes dock at the

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ER membrane such that the nascent translation product is translocated into the ER lumen, followed by cleavage of its signal peptide.[16,17] Upon entry into the ER lumen, proinsulin becomes exposed to an oxidizing environment in which cysteine thiols are highly reactive.[18] Proinsulin contains only six cysteine residues,[19] and, like most members of the insulin superfamily, proinsulin forms three evolutionarily conserved native disulfide bonds (Fig. 1), including proinsulin Cys(B7)–Cys(A7), Cys(B19)–Cys(A20), and Cys(A6)–Cys(A11).

The first evidence that things might go wrong in proinsulin disulfide pairing in the ER resulted from Tris–tricine–urea–SDS-PAGE analysis of proinsulin variants bearing genetically engineered S(B9)D, H(B10)D, or V(B12)E substitutions: all proinsulin molecules migrated similarly under reducing conditions, but a significant subfraction of the variant proinsulin molecules migrated as an anomalously shifted (less compact) form under nonreducing conditions.[20] Additionally, it was noted that one contribution of the C-peptide is to permit the proinsulin polypeptide chain to fold back on itself, such that genetically engineered foreshortening of the C-peptide interfered with the B-chain properly aligning with the A-chain for formation of the Cys(B7)–Cys(A7) and Cys(B19)–Cys(A20) disulfide bonds.[21]

In the course of these analyses, the observation emerged that small quantities of nonnative proinsulin disulfide isomers were also formed during the synthesis of wild-type proinsulin in isolated, normal pancreatic islets,[22] and the most predominant non-native form in these islets showed selectively increased recognition by the ER HSP70 chaperone known as BiP (Fig. 2); this nonnative form was recovered at increased levels from islets incubated under high-glucose conditions[22] that promote translatability of the preproinsulin mRNA.[23] Thus, production of a small fraction of misfolded proinsulin monomers occurs as a natural by-product in the synthesis of native proinsulin.

Proinsulin dimerizes[24] within the ER,[25,26] and this has interesting ramifications in cells expressing misfolded proinsulin that remains trapped in this compartment because of ER quality control.[27] Indeed, when expressing a proinsulin that is C-terminally tagged with KDEL (to prevent successful anterograde trafficking because of retention/retrieval via KDEL receptors), co-expressed untagged proinsulin is retained in *trans*, whereas some unrelated secretory proteins can continue to proceed unimpeded through the secretory pathway.[28] These data indicate that an ER-retained proinsulin can transfer the property of being retained (i.e., unsuccessful anterograde traffic) to other bystander proinsulin molecules within the ER.

The *Akita* mouse develops beta cell failure and diabetes caused by misfolded

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proinsulin. Interestingly, this animal expresses three wild-type *INS* alleles and a fourth allele encoding mutant proinsulin-C(A7)Y that is retained in the ER and triggers ER stress.[29,30] However, because ER-retained proinsulin can transfer its retention property to bystander proinsulin molecules, *Akita*-proinsulin causes co-retention of endogenous wild-type proinsulin.[31] Curiously, by SDS-PAGE and western blotting, a (GFP-tagged) *Akita* proinsulin was predominantly recovered under nonreducing conditions not as a monomer but as a disulfide-linked complex (Fig. 3A, arrows). Immunoprecipitation of tagged *Akita* proinsulin co-precipitates endogenous (untagged) bystander proinsulin molecules that also become engaged in disulfide-linked protein complexes but can be recovered as a monomer upon SDS-PAGE under reducing conditions (Fig. 3B).

Increasing evidence suggests that an abundance of disulfide-linked proinsulin aggregates is likely to contribute to beta cell ER stress and diabetes, thereby raising the question: What are the factors that promote increased abundance of disulfide-linked proinsulin aggregates? We reason (Fig. 4) that, in the steady state, proinsulin aggregates accumulate on the basis of (1) the rate of their formation that is linked to the rate of proinsulin synthesis, which is upregulated in states of increased metabolic demand; (2) the prevailing ER environment, which may not be homeostatically maintained to provide both optimal oxidative capacity and functional helper proteins for proinsulin folding; (3) the presence or absence of primary structural defects intrinsic to the proinsulin molecule itself; and (4) the disposal (or, conversely, the stability/resistance to disposal) of both misfolded proinsulin monomers and aggregates, primarily by ER-associated degradation (ERAD). Both the formation and prevention of accumulation of these aggregates are the subjects of the current review.

Misfolded proinsulin molecules occur in conjunction with proinsulin synthesis

In addition to increased proinsulin synthesis observed upon metabolic demand leading to increased abundance of misfolded proinsulin monomers,[22] there is an additional increase of proinsulin synthesis if islet beta cells fail to deliver sufficient signaling from one of their major ER stress sensors, known as PERK.[32] PERK is a negative regulator of general translation and is highly expressed in islets. Deficiency of pancreatic PERK-mediated phosphorylation of eIF2α, the regulatory subunit (that controls the guanine nucleotide exchange activity) of eIF2B, results in enhanced proinsulin synthesis that is detectable at all

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glucose levels but may contribute particularly under prolonged hyperglycemic conditions, in which increased ER stress response, including eIF2α phosphorylation, would normally be expected.[32] Conversely, a dephosphorylated state of eIF2α has been reported to be stimulated by short-term increases in extracellular glucose,[33] and this correlates at least partially with increased protein synthesis in beta cells[34] (Fig. 5). Loss of PERK function with an exuberant proinsulin synthetic response, even at lower glucose levels, has been directly linked to proinsulin misfolding. PERK deficiency in beta cells causes ER distention[32,35] with proinsulin abnormally retained therein.[36,37] Indeed, when pancreatic beta cells are lysed, and the detergent-solubilized cytoplasmic proteins are spun for 450,000 *g*-min onto a 20% glycerol cushion, proinsulin does not ordinarily penetrate the glycerol cushion. However, within a few hours after beta cells are treated with a chemical inhibitor of PERK, proinsulin synthesis is augmented, and the proinsulin that begins to accumulate in these cells is able to pellet through the glycerol cushion, indicating the formation of abnormally high-molecular-weight protein complexes.[38] When analyzed by SDS-PAGE under nonreducing conditions, some of the proinsulin (molecular mass ~ 9 kDa) can be recovered in complexes up to ~100 kDa—although the same protein is recovered at its usual expected molecular mass by SDS-PAGE under reducing conditions.[38] Moreover, the accumulated proinsulin that was synthesized at increased levels under PERK-deficient conditions is defective for anterograde trafficking to the Golgi complex,[39] leading to deficient insulin production and secretion. This behavior appears to explain the phenotype of patients with Wolcott-Rallison syndrome,[40] a disease caused by naturally occurring loss-of-function mutations in *PERK* leading to neonatal diabetes attributable to beta cell failure[32] as well as multiple other tissue defects.[41]

PERK is one of four kinases that can specifically phosphorylate eIF2α at Ser51 and thereby negatively regulate global protein synthesis as part of the integrated stress response.[42] Defects in eIF2α phosphorylation can be emulated by expression of the eIF2α-S51A mutant that can no longer be phosphorylated at this site. Global eIF2α-S51A heterozygous animals have been found to be sensitive to high-fat diet–induced diabetes with diminished insulin secretion accompanied by distention of the ER lumen in pancreatic beta cells (similar to that seen in PERK deficiency) along with increased proinsulin binding to the HSP70 family member of the ER (known as BiP) in conjunction with defective anterograde proinsulin trafficking.[43] Global homozygous eIF2α-S51A expression (i.e., unregulated overactivity of the eIF2B complex) is a lethal condition[44] that can be rescued by global expression of a wild-type floxed eIF2α transgene, which restores eIF2α phosphorylation and negative regulation of

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proinsulin synthesis in islet beta cells and prevents lethality. In such animals, superimposed beta cell–specific expression of Cre recombinase deletes this transgene (in beta cells), causing proinsulin synthesis to increase in a manner that cannot be suppressed despite ER stress—and, once again, this leads to distention of the beta cell ER accompanied by insulin-deficient diabetes.[45] All of these findings suggest that excessive synthesis of proinsulin promotes its misfolding to a level that impairs insulin production, leading to insulin deficiency. Could excessive proinsulin biosynthesis predispose to beta cell dysfunction in type 2 diabetes? Using *db/db* mice as a model, the answer is yes, as it has recently been demonstrated that glucose-stimulated proinsulin biosynthesis is 14- to 15-fold increased over that in otherwise congenic animals lacking the *db* allele (basal proinsulin biosynthesis is also increased, as is the intracellular proinsulin-to-insulin ratio), and the increased proinsulin no longer exhibits a typical Golgi-like distribution pattern but rather a steady-state distribution suggestive of enhanced accumulation in the ER.[46] Of course, the relevance of these findings to beta cell failure in human obesity–related type 2 diabetes still remains to be proven.[47]

Curiously, in contrast with loss of PERK-mediated phosphorylation of eIF2α, activation of PERK-mediated phosphorylation of eIF2α, despite initial protein synthesis repression, can ultimately lead to downstream activation of transcription factors ATF4, CHOP, and GADD34, which themselves may promote a rebound increase of protein synthesis through enhanced amino acid uptake, tRNA synthesis, and even dephosphorylation of eIF2α[48–50]—these rebound conditions can also potentially add to cellular stress and protein misfolding. Additionally, increased glucose stimulates the Ire1-spliced XBP pathway to expand ER capacity and accelerate proinsulin translation[51] while also promoting the formation of ubiquitylated protein aggregates in beta cells.[52]

Misfolded proinsulin molecules occur in conjunction with perturbations of the ER folding environment

Accumulation of misfolded proinsulin is thought to occur under conditions of molecular crowding within the limited ER luminal space.[53,54] The ER luminal environment has been estimated to bear a protein concentration of ~ 100 mg/mL; such a concentration could not permit proinsulin solubility without the support of ER-resident chaperone and foldase activity.[55] Presumably, beta cells are more optimized than other cells to promote proinsulin

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folding and solubility, as recombinant proinsulin expression in heterologous cell types is thought to fare worse than endogenous proinsulin expressed in beta cells.[56] Nevertheless, as described throughout this review, beta cells remain quite susceptible to proinsulin misfolding. In the last decade, it has been suggested that a portion of proinsulin in beta cells may be recovered in non-native oligomers or aggregate, the formation of which is susceptible to disturbances in beta cell energy production, calcium changes, reductive stress, and inflammatory cytokine exposure.[57] The relative abundance of misfolded proinsulin molecules, through dominant-negative behavior, further influences the success of folding of additional bystander proinsulin molecules.[28] Additionally, several resident proteins have been found to be more abundant in the ER of glucose-responsive (compared with unresponsive) beta cells, including ERp29 (PDIA9), protein disulfide isomerase (PDI), BiP, and the HSP90 family member of the ER known as GRP94.[58] Each of these proteins has been found by ER-specific proteomic analyses of pancreatic beta cells.[59] An area of intense interest involves treatments that may allow beta cells to alter their ER luminal environment in ways that may allow for improved function in the face of increased misfolded proinsulin.

One of the most straightforward ways to document success or failure of proinsulin folding to the native state is by evaluating the status of its three disulfide bonds.[15] There is evidence to support the view that formation of these disulfide bonds may be catalyzed directly or indirectly within the ER environment.[60,61] Peroxiredoxin-4 (PRDX4) has the potential to contribute to proinsulin disulfide bond formation, but ordinarily its expression level in pancreatic beta cells is thought to be very low.[62] One of the main ER oxidases that functions upstream of ER oxidoreductases is ER oxidoreductin 1 (ERO1). First discovered in yeast cells, ERO1 can receive reducing equivalents from PDI, which in turn can receive reducing equivalents from the cysteine thiols of substrate proteins.[63] Pancreatic beta cells express two ERO1 homologs: the ubiquitously expressed ERO1α and the more narrowly expressed ERO1β;[64] evidence suggests that ERO1 activity participates in proinsulin folding,[65,66] and loss of ERO1β gene expression exacerbates the disease phenotype observed in *Akita* diabetic mice[65] (as noted above, these animals express wild-type proinsulin from three normal alleles and misfolded proinsulin-C(A7)Y from one mutant *Ins2* allele[30, 67]).

In the oxidation chain downstream of ERO1 are a wide array of ER oxidoreductases.[68] Whereas PDI is the flagship member, and PDI has been found to help beta cells overexpressing human IAPP,[69] evidence is still lacking that PDI participates directly in

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productive proinsulin folding.[70] Indeed, overexpression of PDI in cultured beta cells promotes ER accumulation of proinsulin and decreased glucose-stimulated insulin secretion.[71] However, oxidized PDI may still be valuable in the ER to oxidize other oxidoreductases of the ER lumen; indeed, in equilibrium, one ER oxidoreductase may distribute its oxidation power to the other intraluminal ER catalysts of disulfide bond formation.[72,73] Moreover, rather than playing a direct role in proinsulin folding, PDI may participate in ERAD of proinsulin[74] in conjunction with other ER luminal components.[75] As discussed further below, this could have significant indirect consequences for the folding of newly synthesized proinsulin in the ER of pancreatic beta cells.

Two other major ER oxidoreductases—P5 (PDIA6) and ERp46 (PDIA15)—have been reported to be preferential oxidation substrates for PRDX4[73] (which is not thought to be highly expressed in beta cells), yet they may have potential to promote insulin production.[76,77] The action of P5 (PDIA6) in beta cells might not involve a direct effect on proinsulin folding[78] and might similarly be involved in proinsulin ERAD[79] or in physiological ER stress signaling via interactions with IRE1.[80] ERp44 (PDIA10) has also been suggested to be a proinsulin-interacting protein,[81] but its function in proinsulin folding is unknown. Additionally, ERp72 (PDIA4) has been implicated indirectly in proinsulin misfolding.[82]

ER molecular chaperones (lacking oxidoreductase activity) have also been strongly linked to proinsulin folding. The ER luminal chaperone BiP is not only upregulated in response to beta cell ER stress, but BiP is routinely found in complexes with misfolded proinsulin molecules.[30,83] Transgenic BiP overexpression in islet beta cells did not change islet insulin content but did protect against high-fat diet–induced diabetes, suggesting improvement in proinsulin folding.[84] BiP overexpression similarly improved glucose-stimulated insulin secretion in cultured beta cells exposed to chronic hyperglycemia.[71] BiP activity to promote insulin production in beta cells is likely to be supported by helper proteins, such as co-chaperone ERdj4,[85] the nucleotide exchange factor SIL1,[86] the DnaJ homolog p58IPK,[87] and GRP170 (discussed below).

ER calcium concentration is another homeostatically regulated parameter of the ER luminal environment that is attuned to optimize the performance of many ER-resident proteins. Cytokine activity can result in the depletion of ER luminal calcium and can activate ER stress[88] that may be mediated by proinsulin misfolding—possibly because ER luminal calcium deficiency creates secondary deficiency in the maintenance of the ER oxidative environment needed for disulfide bond formation.[89] Cytosol-to-ER lumen calcium pumping

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in various tissues is driven by members of the SERCA protein family,[90] of which SERCA2b is a major contributor to ER calcium as well as a contributor to the differentiated function of beta cells,[91,92] which may account for the observation that thapsigargin, a pan-SERCA pump inhibitor, impairs proinsulin export from the ER[93] and induces beta cell ER stress.[94]

Other resident proteins in the beta cell ER luminal environment are less well defined but may also be important, including SDF2L1, which may affect the ERAD rate of misfolded proinsulin,[95] as well as numerous other proteins, such as Torsin 1–3, that are present in the beta cell ER[59] but remain uncharacterized outside of the nervous system.[96]

Overall, the ER luminal microenvironment is homeostatically regulated via physiological ER stress-response signaling, also known as the unfolded protein response (UPR). A detailed discussion of UPR signaling is beyond the scope of this review, but there is reason to think that each of the major ER stress–sensor proteins contributes to fine-tuning the composition of the proinsulin folding environment. Adult mice lacking IRE1 in beta cells exhibit normal general secretory pathway morphology and normal proinsulin mRNA levels but decreased proinsulin synthesis, with some ER-resident protein mRNAs decreased as well (such as ERdj4), plus decreased *ATF6* (at the mRNA level) and spliced *XBP1*—these are all genes needed for adequate beta cell biosynthetic response to glucose—and the net result is diminished insulin secretory granules leading to hypoinsulinemia and hyperglycemia.[51,97,98] Mice lacking XBP1 (downstream of IRE1) in beta cells also exhibit impaired proinsulin processing, decreased insulin content in beta cells, and hyperglycemia.[99] Mice lacking ATF6 exhibit a swollen beta cell ER, accompanied by diminished pancreatic insulin content.[100] And, as noted above, mice deficient for PERK in beta cells develop a swollen ER with accumulated proinsulin and diabetes.[32]

Misfolded proinsulin molecules occur in conjunction with proinsulin coding sequence mutations

Based on proton NMR and additional biophysical analyses, the solution structure of proinsulin reveals a folded insulin moiety (sharing the same three disulfide bridges as that of native insulin) and a largely unstructured connecting peptide, except at the cleavage junctions.[101] Two of the three disulfide bonds of proinsulin are largely buried within the globular protein, whereas the Cys(B7)–Cys(A7) bond lies closer to the protein surface.[102] Within the ER, formation of the latter disulfide bond demonstrates cooperativity with the

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formation of the critical Cys(B19)–Cys(A20 linkage.[15] In mouse pancreatic islets pulsed with ^{35}S-amino acids, within a 60-s labeling period (and no chase), a large fraction of newly synthesized preproinsulin molecules have completed the nascent chain, cleaved the signal peptide utilized for entry into the ER, and already formed all three of the native proinsulin disulfide bonds (Fig. 6)— thus, the native folding of proinsulin is ordinarily a very rapid process.[25]

A large subset (approximately 30) of the reported *INS* mutations are known to trigger a form of diabetes known as mutant INS gene–induced diabetes of youth (MIDY);[103] which masquerades as several different diabetic syndromes but is often linked to neonatal diabetes (first 6 months of life); about a dozen other mutations lead to diabetes diagnosed after 6 months (sometimes referred to as MODY-10). Published data indicate that all of the distinct MIDY alleles, to greater or lesser degrees, encode a misfolded proinsulin protein.[104] Since humans have only one INS gene (one paternally inherited and one maternally inherited allele), it is a reasonable first approximation that 50% of the proinsulin molecules synthesized in MIDY patients represent the misfolded mutant protein. This fraction of misfolded proinsulin may be much greater than the fraction of proinsulin misfolding caused by mere upregulation of the synthesis of wild-type proinsulin,[22,43] such as may exist in type 2 diabetes.[105] On this basis alone, it should not be surprising that, in families transmitting the mutant gene through the germ line, the disease is inherited as an autosomal dominant (i.e., expression of the heterozygous mutant allele tracks with the diabetes phenotype).[106,107] By contrast, insulin haploinsufficiency is inherited as a recessive trait and thus does not, in single copy, cause insulin-deficient diabetes.[108]

The mutant proinsulin proteins encoded by different MIDY alleles have been characterized in a variety of ways. One method exploits the fact that the C-peptide of proinsulin is an unstructured domain;[101] researchers have taken advantage of this property by inserting an epitope tag or even the entire green fluorescent protein (GFP) into the C-peptide, which allows for normal insulin production in beta cells while providing special recognition of the gene product from the tagged allele.[109] It is not surprising that all of the expressed MIDY mutants, to varying degrees, show an increase in ER localization/accumulation.[31,110,111]

Importantly, the onset of diabetes from the MIDY gene product is attributed to a gain-of-toxic-function the results in diminished insulin production and secretion from the wild-type gene product. In *Akita* mice that carry the proinsulin-C(A7)Y mutation in one of two

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Ins2 alleles, by 4 weeks of age, islet areas are normal, but insulin immunostaining of beta cells is markedly decreased, accompanied by a markedly expanded beta cell ER compartment, misfolded proinsulin associated with BiP, indications of chronic ER stress, decreased islet insulin secretion, and decreased insulin levels in the bloodstream.[30,67,112] The initial decreased islet insulin production occurs because the misfolded proinsulin engages directly in protein complexes with bystander proinsulin molecules, impairing the egress of wild-type proinsulin from the ER and thereby blocking wild-type insulin production.[31]

Eventually, stress in the secretory pathway gives way to progressive perturbation of organelle architecture[113] and beta cell death.[114] Although these may be secondary events, they are no less important; indeed it might be possible to circumvent or suppress some of these secondary phenotypes[87,114–121] even without relief from the primary problem (dominant-negative suppression of wild-type proinsulin anterograde export from the ER). One example of such secondary suppression may be achieved by lowering the body's need for pancreatic insulin by agents that help to lower blood glucose,[122,123] including exogenous insulin.[124,125] Another pharmacologic approach may be to develop compounds that could attenuate proinsulin oligomerization[126,127] and thereby limit dominant-negative effects of misfolded proinsulin on bystander proinsulin molecules.

Misfolded proinsulin clearance

Two main mechanisms of clearance of misfolded proinsulin molecules are ERAD and autophagy. The principles underlying ERAD involve recognition of misfolded proteins, targeting such proteins to the ERAD complex, retrotranslocation across the ER membrane, ubiquitylation of target proteins, extraction of ubiquitylated target proteins from the cytosolic face of the ER membrane, and degradation by 26S proteasomes.[128] The two core molecules of the ERAD machinery are the partner proteins HRD1 and SEL1/HRD3, which are conserved even in yeast.[129] The polytopic HRD1 membrane protein functions in both retrotranslocation and as an E3 ubiquitin ligase, whereas the single membrane–spanning SEL1 physically associates with HRD1 and is required for HRD1 protein stability, while also helping to present ERAD substrates to HRD1; many other ancillary proteins interact with the core complex, and these topics are reviewed in detail elsewhere.[130] The ERAD core complex is upregulated by the ER stress response,[36,131] suggesting that one of its key functions is to help cells adapt to the ER load of misfolded proteins. Failure of the anterograde transport

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pathway carrying proinsulin is a likely trigger for enhanced proinsulin ERAD.[132]

There is strong reason to believe that misfolded proinsulin is an ERAD substrate. First, as noted above, islet beta cells that have misfolded proinsulin exhibit upregulated expression of HRD1 and SEL1/HRD3.[133,134] Second, treatment of beta cells with a variety of perturbants that poison the ER folding environment has been found to increase both proinsulin misfolding and proinsulin disappearance by pulse-chase analysis.[57,135] Third, many MIDY mutant proinsulins have been shown to be degraded, at least in part, via proteasomal disposal mechanisms.[104] Fourth, short hairpin RNA (shRNA)-mediated knockdown of HRD1 and the p97 ATPase (involved in ER extraction of ubiquitylated proteins for proteasomal disposal) were both reported to increase steady-state levels of proinsulin in beta cells.[136]

Recent studies of the proinsulin-C(A7)Y mutant, which causes diabetes in the *Akita* mouse, have confirmed that the misfolded proinsulin utilizes the HRD1–SEL1L core ERAD complex and further requires p97 for its proteasomal degradation.[74] Intriguingly, PDI was also found to engage the misfolded mutant proinsulin, and the absence of PDI activity triggered enhanced proinsulin aggregation into aberrant disulfide-bonded proinsulin complexes, clarifying one of the upstream elements involved in recognition or targeting misfolded proinsulin to the ERAD core complex.[74] Additional work has revealed that the ER chaperone GRP170 also acts in the upstream ERAD pathway of misfolded proinsulin in such a way as to decrease the abundance of high-molecular-weight proinsulin aggregates, promoting smaller proinsulin complexes that have better competence for retrotranslocation and proteasomal disposal.[75] Interestingly, GRP170 is particularly highly expressed in islet beta cells, and its expression seems to parallel that of proinsulin.[137] Most remarkable, in cells expressing both wild-type proinsulin and the proinsulin-C(A7)Y mutant, GRP170 overexpression assisted in the selective degradation of the mutant, liberating the wild-type proinsulin for export from the ER and production of native insulin.[75] This is of obvious potential interest in limiting proinsulin aggregation in order to improve beta cell function for the prevention of diabetes.

There is also reason to suspect that ER-phagy may be an important pathway of misfolded proinsulin disposal.[7] First, proinsulin misfolding has been observed to stimulate autophagy.[117] Second, mice with beta cell-specific deletion of the ATG7 autophagy gene showed accumulation of intracellular ubiquitinated protein aggregates and ER distention associated with hypoinsulinemia, decreased pancreatic insulin content, and decreased beta

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cell mass leading to hyperglycemia.[138] Third, treatment of female *Akita* mice with rapamycin (an mTOR inhibitor), which stimulated autophagosome formation and autophagic flux in beta cells, also increased pancreatic insulin content and decreased measures of beta cell ER stress.[139] However, proinsulin that has undergone anterograde trafficking beyond the ER may also reach autophagosomes, and such molecules are unlikely to be misfolded.[140]

Clinical implications

Type 2 diabetes is the most common form of diabetes mellitus worldwide, and it is still debated whether stimulating beta cell insulin secretion or providing beta cell rest is the optimum therapy for the disease.[141] Before onset of disease, beta cells increase their insulin production in response to increased demand driven by insulin resistance. Under states of pancreatic insulin depletion, increasingly active beta cell exocytosis is also accompanied by hyperproinsulinemia,[142,143] which decreases available substrate for conversion to insulin and adds more pressure on beta cells to increase active hormone production through proinsulin biosynthesis. Exposure to cytokines[144,145] and environmental toxins[146] could further exacerbate this problem (Fig. 7). Both hyperlipidemia and hyperglycemia may initially promote more proinsulin biosynthesis, but they ultimately trigger severe beta cell ER stress.[147]

It is generally recognized that type 2 diabetes does not develop until there is a significant deficiency of insulin secretion.[148] While the initiating/inciting origin of this common insulin secretory defect remains obscure in garden-variety type 2 diabetes, evidence from rare (monogenic) forms of diabetes are shedding light on basic processes essential for healthy insulin production. Indeed, even as complex diseases, such as diabetes, have a multifactorial etiology (including both genetic and environmental predisposition), a number of rare forms of diabetes exhibiting Mendelian inheritance (including autosomal dominant inheritance) may provide highly fruitful clues to the intracellular organelles and pathways that are most susceptible to breakdown in beta cell dysfunction. Some of these rare diseases include Wolfram syndrome,[149] which involves a protein (WFS1) that influences the beta cell ER environment;[150–152] Wolcott-Rallison syndrome,[41,153] which, as noted above, causes loss of PERK function in the ER and thereby tends to enhance preproinsulin translation;[154] and MIDY,[103] which is a direct cause of proinsulin misfolding in the ER and insulin-deficient diabetes.[7]

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In garden-variety type 2 diabetes, it is increasingly recognized that genetic perturbation of islet regulatory elements can alter islet transcriptional responses to ER stress,[155] to which beta cells are highly susceptible.[156] Genome-wide association studies may also suggest that allelic variants encoding proteins involved in ER-related processes can confer increased type 2 diabetes susceptibility.[157] Indeed, islet beta cells from human type 2 diabetes patients have an increased ER volume density that is plausibly consistent with increased proinsulin synthesis; at the same time, isolated islets from type 2 diabetic individuals exhibit decreased insulin secretion, and these islets exhibit a more easily induced ER stress response to hyperglycemic conditions.[158]

Increased proinsulin production in type 2 diabetes accompanied by decreased intrapancreatic insulin stores is also present in animal models.[159] There is increasing evidence of islet beta cells with vigorously active ongoing proinsulin biosynthesis that are nevertheless insulin deficient (by immunofluorescence).[46] There is also evidence that attenuating beta cell proinsulin synthesis can improve proinsulin maturation, insulin production, and insulin secretion *in vivo*[124] and *in vitro*.[46] However, additional work is still needed to establish a direct link between proinsulin misfolding and pancreatic beta cell failure in human type 2 diabetes and to develop therapies to limit accumulation of misfolded proinsulin.[66,75]

In this review, we highlighted features that promote proinsulin misfolding, including its increased synthesis, altered ER environment, the presence of proinsulin structural defects, and deficiency of clearance of misfolded proinsulin. We propose that these processes are fertile areas for studies of molecular and cellular mechanisms that may predispose to beta cell failure during the onset and progression of diabetes mellitus.

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Competing Interests

The authors declare that we have no competing interests related to this work.

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Figure legends

Figure 1. Pattern of disulfide bond formation throughout the insulin/IGF superfamily. (A) Reproduced from Ref. 19, with permission from the publisher. (B) The human insulin peptide sequence is inserted into the disulfide pattern shown in panel A. The first two Cys residues fall within the insulin B-chain (in red), and the remaining four Cys residues fall within the insulin A-chain (in blue). The conversion of the single-chain proinsulin to the two-chain insulin molecule involves excision of the connecting C-peptide (shown in orange) via endoproteolytic cleavage followed by carboxypeptidase cleavage at dibasic sites (green letters).

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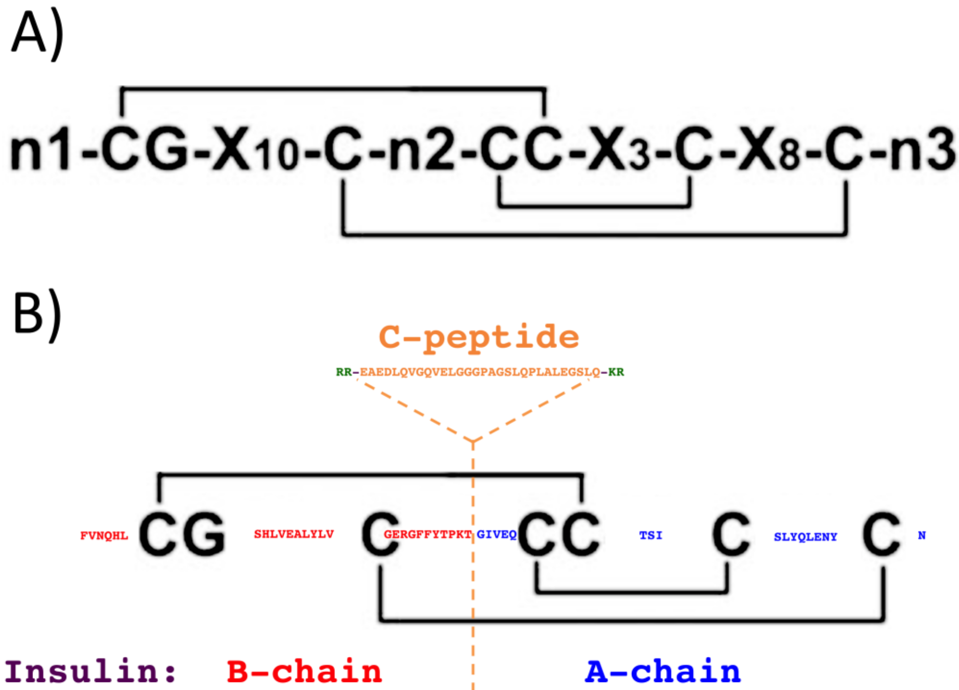

Figure 2. Non-native isomer of newly synthesized proinsulin shows enhanced binding to the ER HSP70 chaperone BiP. Fifty isolated rat pancreatic islets were pulse labeled with ^{35}S-labeled amino acids and then lysed immediately and either immunoprecipitated with a polyclonal antibody to insulin that recognizes all proinsulin forms or co-immunoprecipitated with anti-BiP. The immunoprecipitates were analyzed by nonreducing Tris–Tricine–urea–SDS-PAGE. In addition to native disulfide-bonded proinsulin, a more slowly migrating proinsulin disulfide isomer is preferentially associated with BiP. Reproduced from Ref. 15 with permission from the publisher.

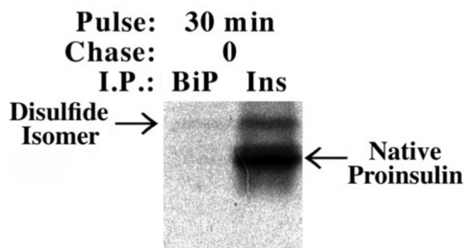

Figure 3. Protein interactions of proinsulin-C(A7)Y. (A) The INS1 pancreatic beta cell line was used, either untransfected (control) or transfected to express hPro-CpepGFP or hProC(A7)Y-CpepGFP (the latter bearing the *Akita* proinsulin mutation). Cell lysates were subjected to western blotting with anti-GFP after SDS-PAGE under reduced or nonreduced conditions. Not only is hProC(A7)Y-CpepGFP not endoproteolytically processed in beta cells, but the protein is recovered in higher-molecular-mass protein complexes (open arrows) that are detected only under nonreduced conditions. (B) The same cells from panel A were pulse labeled with ^{35}S-labeled amino acids for 30 min and lysed, then GFP-containing peptides were immunoprecipitated, and the samples were analyzed by Tris–tricine–urea–SDS-PAGE under reducing conditions to detect coimmunoprecipitation of endogenous proinsulin. Reproduced from Ref. 24 (© 2007; National Academy of Sciences).

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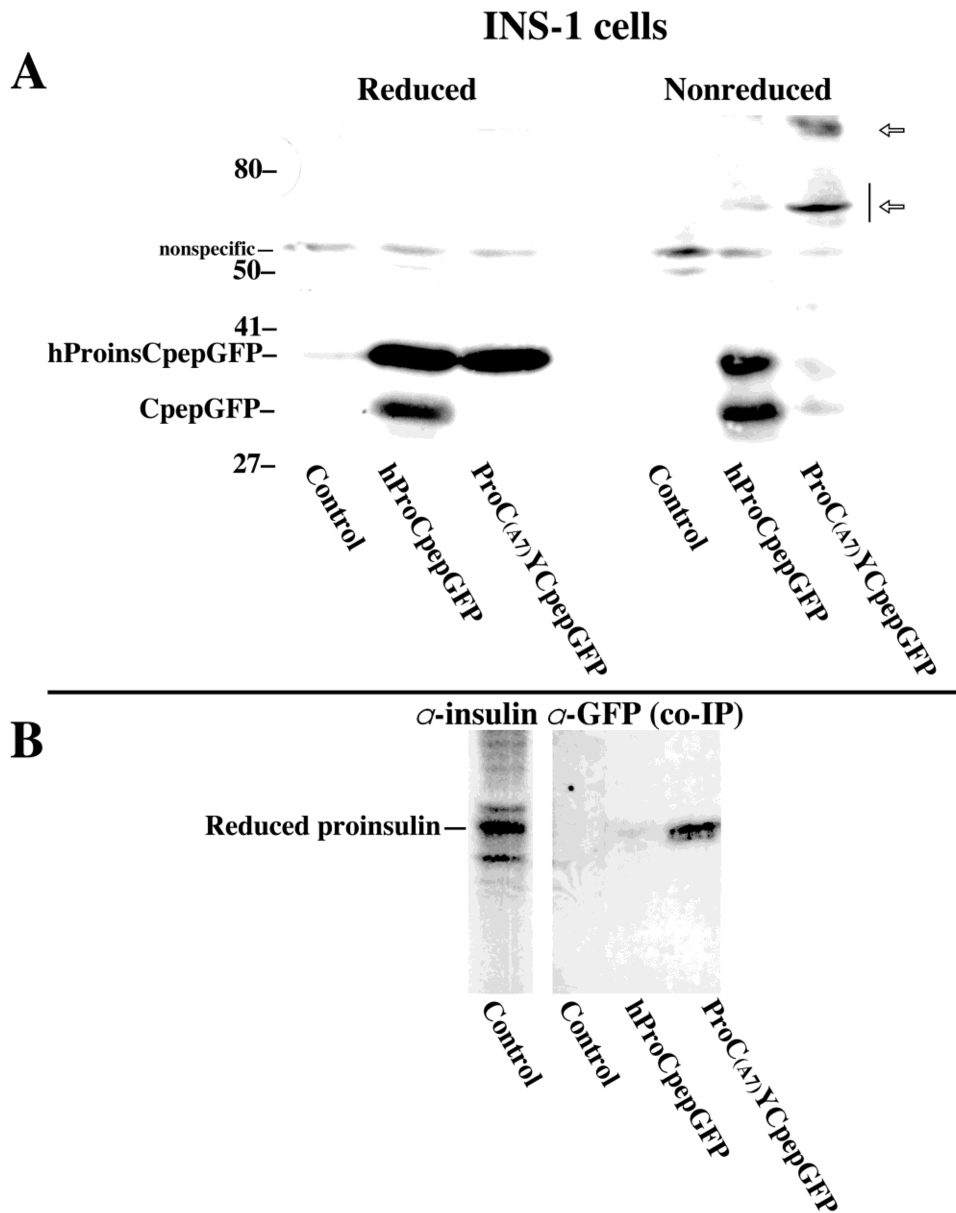

Figure 4. Steps contributing to proinsulin aggregation in the ER folding environment. Upper panel: under healthy conditions, proinsulin synthesis is limited to physiological levels (small font) and folds within a generally favorable folding environment leading to successful export from the ER (thick green arrow). The misfolded proinsulin that is generated in parallel with native folding is actively disposed of, including monomer disposal (thick brown arrow) and aggregate disposal (thick blue arrow). Through each of these mechanisms, the steady-state level of misfolded proinsulin is held to low levels. Lower panel: under unhealthy conditions, proinsulin synthesis is exuberant to a level that may be considered supraphysiological; the increased supply of unfolded monomers leads to the production of more proinsulin aggregates, exceeding the disposal of misfolded proinsulin, such that the steady-state level of misfolded proinsulin is increased.

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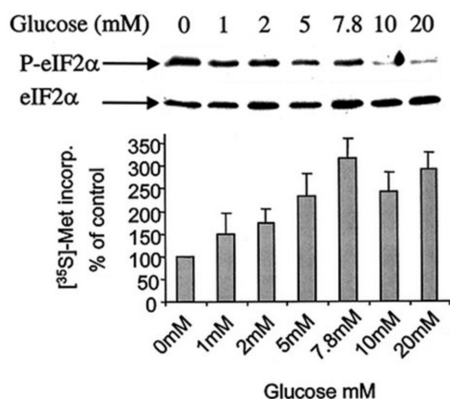

Figure 5. Glucose-related changes in eIF2 phosphorylation in MIN6 cells. MIN6 cells (i.e., cells of a mouse pancreatic beta cell line) were preincubated in no glucose (1 h) before incubation in the indicated concentrations of glucose for 1 h in the presence of [^{35}S]methionine. Upper panel: cell lysates were analyzed by SDS-PAGE and western blotting with anti-phospho-eIF2α (P-eIF2α) and anti-eIF2α. Lower panel: incorporation of [^{35}S]methionine into total protein, as a percent of control (0 mm glucose). Adapted from Ref. 27 with permission from the publisher.

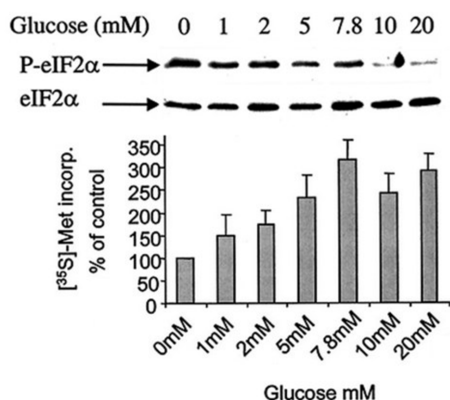

Figure 6. Rapid formation of native proinsulin disulfide bonds. Isolated mouse pancreatic islets were pulse labeled for 60 s with ^{35}S-labeled amino acids and either lysed without chase or chased in the presence of cycloheximide (100 μg/mL) to prevent further proinsulin synthesis. The islets were lysed and analyzed by Tris–-tricine–urea–SDS-PAGE and phosphorimaging; the position of oxidized native proinsulin is shown. Reproduced from Ref. 18 with permission from the publisher.

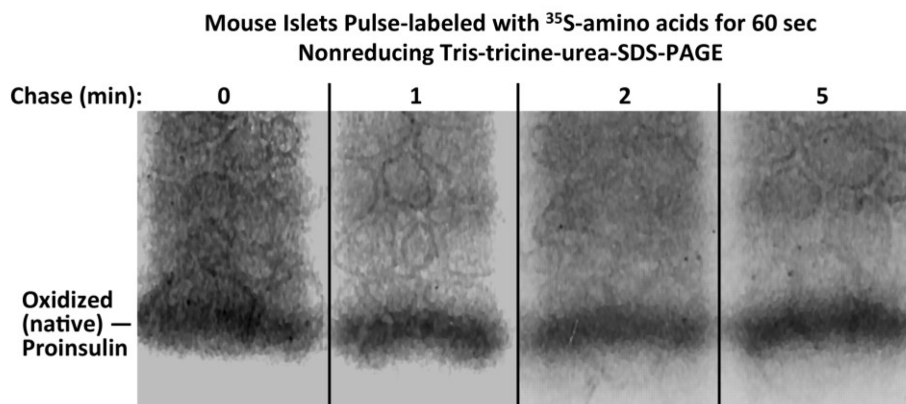

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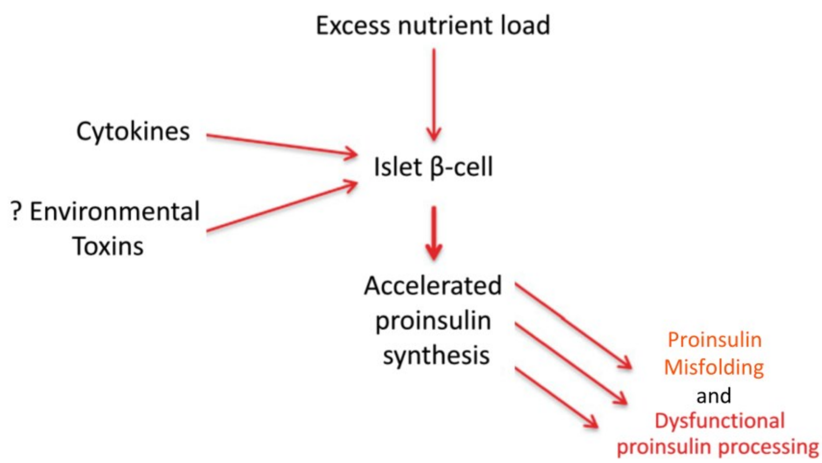
Figure 7. Hypothesis: relevance of proinsulin misfolding to insulin deficiency/beta cell dysfunction in garden-variety diabetes mellitus. This figure has been adapted and altered from Fig. 1 of Ref. 47; permission from publisher is pending.



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