Published in final edited form as:

Trends Endocrinol Metab. 2010 November ; 21(11): 652–659. doi:10.1016/j.tem.2010.07.001.

Proinsulin misfolding and diabetes: *Mutant INS* gene-induced Diabetes of Youth

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Abstract

Type 1B diabetes (typically early onset; without islet autoantibodies) has been described in patients bearing small coding sequence mutations in the *INS* gene. Not all mutations in the *INS* gene cause the autosomal dominant *Mutant INS*-gene-induced *Diabetes of Youth (MIDY)* syndrome, but most missense mutations affecting proinsulin folding produce *MIDY*. *MIDY* patients are heterozygotes, with the expressed proinsulin mutants exerting dominant-negative (gain of toxic function) behavior in pancreatic beta cells. Herein, we focus primarily on proinsulin folding in the endoplasmic reticulum, providing insight into perturbations of this folding pathway in *MIDY*. Accumulated evidence indicates that in the molecular pathogenesis of the disease, misfolded proinsulin exerts dominant effects that initially inhibit insulin production, progressing to beta cell demise with diabetes.

Basics of proinsulin structure and risk of misfolding in the ER

Despite a net weight in humans of only ~2 grams, pancreatic islets and their secreted peptide hormones are essential for normal body metabolism. This is especially true of glucose-regulated insulin production and insulin secretion from pancreatic beta cells. The protein production pathway begins when the *INS* gene product is translated as preproinsulin¹. From N- to C- terminus, the precursor is comprised sequentially of the signal peptide (SP, residues 1-24), insulin B-chain (residues 1-30), C-peptide (residues 1-31) plus its two sets of flanking dibasic cleavage sites, and the insulin A-chain (residues 1-21). The newly-synthesized preproinsulin signal peptide facilitates recruitment to the endoplasmic reticulum (ER), whereupon the growing nascent polypeptide is cotranslationally translocated across the ER membrane into the ER lumen.

Approximately half of daily insulin secretion accommodates ongoing anabolic needs under basal conditions between meals, while the other half is secreted in response to eating². Even with the substantial decrease in insulin expression and secretion under fasting conditions, proinsulin biosynthesis still dominates the beta cell proteome³. Proinsulin monomer folding and dimerization is thought to occur within the ER lumen; traffic through the Golgi complex and into immature secretory granules brings proinsulin in contact with zinc facilitating its

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further assembly to hexamers⁴. Ultimately, proteolytic excision of the C-peptide and its dibasic flanking residues, along with organelle remodeling, results in insulin storage in mature secretory granules^{5, 6}.

Much is known about proinsulin or insulin folding *in vitro* while relatively little is known about proinsulin folding *in vivo*⁷. Indeed, experiments have not yet established whether the following processes are strictly sequential events: 1) completion of translocation, 2) excision of the signal peptide catalyzed by the ER signal peptidase, 3) initiation of B-chain folding, and 4) initiation of disulfide bond formation leading to the three evolutionary conserved disulfide bonds of proinsulin [destined to be ‘interchain’ within insulin: C(B7)-C(A7) and C(B19)-C(A20), as well as intrachain C(A6)-C(A11)]. In the ER, these events are estimated to occur within a time span of 20 seconds to perhaps a minute from the initiation of preproinsulin translocation. Given the competition between reaction rates in pancreatic beta cells and all eukaryotic cells, it is likely that within a cohort of nascent preproinsulins or other secretory proteins, molecules may engage in closely-spaced early events in a stochastic process⁸, i.e., with a sequence of events that are not precisely identical between major and minor subpopulations of preproinsulins.

With regard to initiation of B-chain folding, the secondary structure of proinsulin includes three alpha-helical domains. Based on proinsulin delivery into the ER lumen from N- to C-terminus, the B-chain has the earliest folding opportunity, and acquisition of the central B-chain alpha helix running from S(B9) - C(B19), including side-chain packing at residue V(B12)9, may be an early event to facilitating formation of the C(B19)-C(A20) disulfide bond and driving the folding pathway forwards¹⁰. In conjunction with additional B-chain structural features¹¹, an N-terminal A-chain alpha-helix including V(A3) - A(A8) includes alignment of the C(B7)-C(A7) and C(A6)-C(A11) disulfide bonds while a C-terminal A-chain alpha helix including L(A13) - Y(A19) influences alignment of C(B19)-C(A20)¹²⁻¹³. Within the ER luminal environment, proinsulin disulfide bond formation is coupled to the reduction of one or more resident ER oxidoreductases¹⁴, which are in turn catalytically recycled via their own reduction of Ero1beta and/or Ero1alpha¹⁵⁻¹⁶.

Formation of disulfide bonds within proinsulin that are destined to be ‘interchain’ within insulin, i.e., C(B7)-C(A7) and C(B19)-C(A20), are essential for stabilizing a three-dimensional structure suitable for anterograde export of the prohormone from the ER¹⁷. Within the three-dimensional structure of proinsulin, hydrophobic core residues are buried in the interior of the molecule¹², whereas the C(B19)-C(A20) disulfide bond is not totally buried, and the C(B7)-C(A7) bond is nearly fully exposed on the surface of the folded polypeptide¹⁸, so as to render proinsulin susceptible to destabilization by thiol attack.

As much as a third of all newly synthesized proteins may fail to fold properly, requiring either refolding or elimination¹⁹; thus, the “substrate” for proinsulin misfolding already exists in the wild-type molecule²⁰. In the event of proinsulin misfolding, “sticky” residues from the hydrophobic core and unpaired or mispaired cysteine thiols create significant risk for non-native intermolecular interactions with nearest neighbors including the high concentration of bystander proinsulin molecules in the ER. Such interactions could confer ER retention on bystander proinsulin molecules, limiting their potential for insulin production.

Evidence for proinsulin misfolding in the ER

The first indication of the possibility of such misfolding emerged upon biosynthetic expression of an insulin analog bearing the P(B28)K,K(B29)P double mutation (clinically known as ‘insulin lispro’), leading to low protein production of the precursor in the ER, with the expressed product containing aberrant disulfide isomers²¹. This was surprising given that *in vitro* folding stability of some monomeric insulin analogs, including lispro, is thought to be comparable to that of native insulin²²⁻²³. One possible explanation was that foreshortening of the C-peptide

in a bioengineered analog might impair chain alignment for disulfide bond formation¹⁷; however, only extreme shortening triggers increased disulfide mismatch and loss of insulin bioactivity²⁴. In fact, aberrant proinsulin disulfide isomers are made even in pancreatic islets isolated from normal rodents in the absence of any agents that induce ER stress, although the abundance of such aberrant isomers is increased under conditions of increased proinsulin synthesis such as high glucose or genetic inability to maintain the phosphorylation state of Ser51 of eukaryotic initiation factor 2- α (eIF2 α)^{20, 25}. To date, the precise disulfide mismatches in proinsulin have not been identified; however, artificially engineered insulin disulfide isomers bearing native-like structural features²⁶⁻²⁸ might possibly correspond to proinsulin disulfide isomers that occur in the ER of pancreatic beta cells.

Accumulation of misfolded proteins in the ER is a cause of (and synonymous with) ER stress. Temporary or chronic imbalance in the proinsulin folding environment in beta cells leads to temporary or chronic ER stress. ER stress is not synonymous with the ER stress response (see Box 1); in normal physiology, the two are linked but in the pathophysiology of diabetes, imbalance between ER stress and the ER stress response may occur. For example, genetic^{29, 30} or epigenetic³¹ incompetence in the ER stress response only increases ER stress²⁵. This has been shown for misfolded secretory proteins other than proinsulin^{32, 33}, and may also occur in states of misfolded proinsulin accumulation such as upon dysregulation of proinsulin biosynthesis^{34, 35} or degradation³⁶. Temporary and chronic ER stress response activation is linked to both pro-survival and pro-apoptotic pathways in pancreatic beta cells, as reviewed extensively by others^{29-31, 37-39}.

Insulinopathies

Most monogenic mutations in the coding sequence of insulin, including classical insulinopathies and *MIDY* mutants (the latter being the focus of this review), result in autosomal dominant disease with a spectrum of severity, ranging from late onset diabetes with hyperinsulinemia to early onset diabetes with insulin deficiency. Additionally, a smaller subset of patients with neonatal diabetes have recessive (loss-of-function) mutations in the *INS* gene, resulting in impaired insulin biosynthesis⁴⁰.

The classical insulinopathies (so named because the mutants have been known for many years), involve three molecular mechanisms: a) poor insulin binding to the insulin receptor with hyperinsulinemia secondary to poor clearance of mutant insulin-V(A3)L, -F(B24)S, or F(B25)L from the circulation⁴¹; b) endoproteolytic cleavage site mutations⁴²; and c) mutant proinsulin-H(B10)D that exhibits altered protein storage in the secretory pathway⁴³. Collectively, each of these mutations results in significant secretion of insulin or proinsulin to the bloodstream, and therefore none are likely to exert their effect exclusively within the secretory pathway of pancreatic beta cells.

Mutant *INS*-gene-induced Diabetes of Youth (*MIDY*)

Over the past three years, 26 new *INS* gene mutations have been associated with a monogenic diabetes syndrome⁴⁴⁻⁵¹ that we are calling Mutant *Ins*-gene-induced Diabetes of Youth (*MIDY*), defined as autosomal dominant diabetes with insulin deficiency in the absence of beta cell autoimmunity. Patients with *MIDY* were previously diagnosed most often as having permanent neonatal diabetes mellitus and less frequently as type 1b (autoantibody-negative) diabetes, MODY or type 2 diabetes. Genetic studies have shown that 80% of the mutations causing *MIDY* occur *de novo* with 20% transmitted from parent to child. *MIDY* is second in frequency only to mutations in the sulfonylurea receptor/Kir6.2 complex as a monogenic cause of permanent neonatal diabetes⁵². Although *MIDY* mutations have been suggested to affect the earliest events of proinsulin biosynthesis in the ER⁷, the precise molecular mechanisms of beta cell failure caused by these mutations are incompletely understood.

Mice have two functional, nonallelic insulin genes (with *Ins2* corresponding to the human *INS* gene on chromosome 11). A heterozygous null genotype at the *Ins2* locus — with or without a homozygous null genotype at the *Ins1* locus — is insufficient to cause diabetes^{53, 54}, yet male *Akita* mice inheriting even one *Ins2* allele encoding the *MIDY* mutant proinsulin-C(A7)Y develop insulin deficient-diabetes after weaning. This suggests that the *MIDY* mutant creates a gain of toxic function. Thus far, the *Akita* mouse, expressing proinsulin-C(A7)Y, is the most studied *MIDY* mutant. In the remainder of the review, we first provide an overview of human preproinsulin mutations causing *MIDY*, and then discuss potential molecular mechanisms of diabetes pathogenesis, relying heavily on data from the *Akita* mouse model.

***MIDY* clinical phenotypes linked to molecular phenotypes**

Although most newly identified *INS* gene mutations cause severe insulin deficient diabetes (Table 1), the broad spectrum of clinical phenotypes suggests that different *MIDY* (pre) proinsulin mutations behave differently. Mutated amino acid residues causing *MIDY* have now been found within all domains of the preproinsulin molecule including the signal peptide, C-peptide and flanking cleavage sites. Over 70% of the mutations are contained within the insulin B- or A-chains^{44-48, 55}. Many of these mutations have implications for the maintenance of normal folding and bioactivity of mature (ie, processed) insulin⁷, but it is highly likely that at least some conserved residues of proinsulin are involved primarily in the proinsulin folding pathway (ie, kinetics effects on achieving a final folded structure) rather than being important for structural stability (or even bioactivity) after normal disulfide pairing has been accomplished (ie, thermodynamic effects on the final folded structure)⁵⁶. Many of the *MIDY* mutants create or eliminate a cysteine residue at different positions along the polypeptide chain, resulting in an odd number of Cys residues with the potential to alter or disrupt normal proinsulin disulfide pairing. Of those mutations not altering preproinsulin cysteine content, most are located at evolutionarily conserved residues suggesting that they are indispensable for normal proinsulin folding. Ultimately, both cysteine and non-cysteine *MIDY* mutants highlight the importance of properly paired proinsulin disulfide bonds to prevent development of diabetes caused by misfolded proinsulin.

Four preproinsulin mutations located either near the N or C-terminal end of the signal peptide are likely to affect one or more of the four earliest events of proinsulin biosynthesis: 1) completion of translocation, 2) excision of the signal peptide catalyzed by the ER signal peptidase, 3) initiation of B-chain folding, and 4) initiation of disulfide bond formation. Preproinsulin-R(SP6)C and -R(SP6)H mutations result in deficiency within the signal peptide of an N-terminal positive charge, which has been shown to play an important role for targeting newly synthesized secretory proteins to the ER⁵⁷. Both of these mutations resulted in an ER stress response when the recombinant preproinsulin mutants were expressed in transfected cell lines, yet mutant preproinsulin molecules were properly delivered to and through the secretory pathway⁵⁸. The translocation efficiency of these mutants, as well as the origin of the ER stress, needs further exploration. Preproinsulin-A(SP24)D mutation disrupts normal signal peptide cleavage¹¹, which is likely to interfere with downstream proinsulin folding and trafficking. Expression in a transfected beta cell line demonstrates proper ER targeting of preproinsulin-A(SP24)D but > 95% of the mutant molecules were retained intracellularly as proteins of increased molecular mass (consistent with uncleaved preproinsulin)⁵⁹. By contrast, another study reported that a meaningful fraction of mutant proinsulin reaches secretory granules, and neither expression of the mutant nor a demonstrably associated ER stress response attenuates secretion of co-expressed wild-type insulin or C-peptide⁶⁰. Thus, the biological mechanisms by which this mutant brings about diabetes require further clarification. Finally, whereas preproinsulin-A(SP23)S mutation was reported recently in a patient originally diagnosed with type 1 diabetes⁶¹, Ser is the native residue in this position of chimpanzee preproinsulin, leaving open the question of whether this substitution is a real contributor to diabetes.

Several new proinsulin mutations have been reported in the C-peptide and its flanking dibasic cleavage sites. The proinsulin-G(C28)R mutant was originally identified in a subject diagnosed with type 1 diabetes⁴⁵. However, a new study reports that the proinsulin-G(C28)R variant can efficiently exit the ER and be released from transfected cells⁵⁹, raising questions about whether this mutation is diabetogenic. Of the two *MIDY* mutations reported at flanking cleavage sites, R(Cpep -2)C and R(Cpep +2)C, the latter is more commonly reported, encoded at a CpG hot spot for mammalian mutations⁴⁴. Unlike other mutations occurring at this same amino acid residue, R(Cpep +2)P, R(Cpep +2)L and R(Cpep +2)H, which cause hyperproinsulinemia diagnosed in adulthood^{42, 43}, most patients with *MIDY* mutations at flanking cleavage sites have early onset insulin deficient diabetes. Direct side-by-side comparison of the recombinant proinsulin-R(Cpep +2)C and proinsulin-R(Cpep +2)L mutants showed a profound inhibition of secretion linked to the mutant Cys residue with no inhibition linked to the mutant Leu residue⁴⁸. This finding underscores the potency of unpaired proinsulin cysteines as a perturbant and highlights the range of clinical severity and age of onset of diabetes associated with different mutant alleles even when they encode distinct amino acids at the same position of the preproinsulin polypeptide chain.

Before proceeding on to a detailed consideration of *MIDY* mutations located within the B- and A-chains that create or delete a Cys residue (represented by proinsulin-C(A7)Y, which is discussed below), we wish to mention a final group of mutants involving missense mutations that neither create nor delete a cysteine yet nevertheless cause *MIDY*⁷. Intriguingly, no such mutations have yet been found in the A-chain. By contrast, multiple mutations have been reported in the B-chain, affecting the highly conserved residues 5, 6, 8, 11, 22, or 23. Residue H(B5) facilitates alignment of the proinsulin C(B7)-C(A7) disulfide partners⁶² and recent evidence suggests that sequence variation at this site is a primary reason for the formation of aberrant disulfide isomers involving C(B7) of IGF-1, a proinsulin homolog¹¹. Invariant residue L(B6), which contributes strongly to the structure of bioactive insulin, has been proposed to play a role in maintaining local structure in the vicinity of the C(B7)-C(A7) disulfide and allows flexibility of the N-terminal region to either join the central B-chain alpha helix or turn from it⁶³. Similarly substitution of residue G(B8)S mutagenizes a site critical to formation of the C(B7)-C(A7) disulfide bond⁶⁴. Perturbations around residue L(B11) also impair insulin structure⁹, and computational biology studies suggest L(B11) and R(B22) as two of the 5 most important residues for the stability of individual insulin monomers⁶⁵, including hydrogen bonding⁴⁶ critical to B-chain/A-chain alignment. Finally, substitution at residue G(B23)V may implicate this residue in rotational flexibility of the proinsulin polypeptide backbone⁶⁶, which might facilitate C(B19)-C(A20) pairing; this is a hypothesis that deserves testing. To summarize, the evidence from this group of *MIDY* mutants points towards the idea that proper B-chain structure and flexibility are essential elements for proper cysteine alignments for disulfide bonding in the proinsulin folding pathway. Failure to achieve these alignments sets off the molecular pathogenesis of the autosomal dominant diabetes known as *MIDY*.

Molecular mechanisms of beta cell failure caused by misfolded preproinsulins: lessons from the *Akita* mouse

Although not fully characterized, progress is being made to advance our understanding of the pathogenesis of diabetes caused by misfolded (pre)proinsulin, using *MIDY* as the most clear-cut example. In brief, we suggest that the disease may be initiated when trafficking of normal proinsulin is adversely affected by co-expressed misfolded mutant proinsulin, resulting in a cascade of additional, overlapping defects that culminate in beta cell demise (Fig. 1). The most detailed results to date come from data using *Akita* mice in which a heterozygous proinsulin-C(A7)Y mutation is identical to a heterozygous mutation causing human *MIDY*.

Immunohistological analysis of the pancreas of male *Akita* mice after diabetes onset excludes

lymphocytic infiltration or islet inflammation as a cause of diabetes, yet there is a decrease in insulin immunostaining ⁶⁷.

From immunohistological analysis in a population of *Akita* males and females at postnatal day 1, there is a normal insulin positive area and islet mass ⁶⁸, suggesting that individuals bearing *MIDY* mutations are not born with a deficiency of beta cells. And yet, at the electron microscope level, the beta cells of these animals at postnatal day 1 exhibit expansion of compartments of the early secretory pathway including the ER, which is accompanied by an increase in mitochondria (suggesting a need for increased energy production) and a decrease in insulin secretory granules ⁶⁸. Normally, beta cells use their biosynthetic machinery to generate a large reserve of accumulated secretory granules that represent the major pool of pancreatic insulin. Thus, given that *Akita* mice have normal insulin sensitivity (and thus no need to secrete more insulin than normal), a morphological decrease in the number of insulin granules on postnatal day 1 suggests that there is already a significant block in insulin production. Indeed, in such animals, the pancreatic content of insulin, while not yet low enough yet to trigger detectable diabetes, has already decreased to less than one-third of that of wild-type controls ⁶⁸.

When *Akita* animals begin to show overt signs of lower body weight and higher blood glucose indicating insulin deficiency after a few weeks of postnatal life, a trend towards a decrease in insulin positive area of the islets first becomes noticeable ⁶⁸. However even within the remaining beta cells there is a decrease in 'steady-state' insulin content that is much greater than can be accounted for by loss of one wild-type allele ⁶⁹. Thus, although age-related progression of diabetes leads ultimately to an obvious loss of beta cell mass ⁷⁰, and the underlying genetic background of the individual clearly influences the severity and progression of the diabetes, it appears that in this representative example of *MIDY*, a block in insulin production is a primary defect, preceding the decline of beta cell mass ⁷¹. At a stage of overt diabetes but prior to massive loss of islets, a loss of insulin production is detected despite supranormal levels of proinsulin biosynthesis from the remaining wild-type alleles, indicating a defect of wild-type proinsulin trafficking to secretory granules ⁷². It is quite possible that secondary effects from the hyperglycemia of diabetes further exacerbate the underlying proinsulin trafficking defects and concomitant ER stress ⁶⁹.

In the case of homozygosity of the *Akita* mutation, animals still have two functional *Ins1* alleles, but the higher level of misfolded proinsulin results in fulminant diabetes within two weeks of life, associated with a dramatic loss of total islet area and a reduction in the islet content of beta cells ⁶⁸. Given the presence of the remaining wild-type mouse *Ins1* alleles, the likelihood is that animals bearing the heterozygous *Akita* mutation probably synthesize less *MIDY* proinsulin than their human counterparts (who have only one *INS* gene) whereas homozygous animals probably synthesize an equal or greater fraction of *MIDY* proinsulin than their human counterparts. A critical lesson from the comparison of heterozygous and homozygous animals is that the actual fraction of total newly-synthesized proinsulin comprised of misfolded proinsulin will have a great impact on the acceleration/progression of diabetes.

If two copies of *Ins1* are sufficient to avoid diabetes ^{53, 54}, how can we account for the insulin deficiency before any loss of beta cell mass? In islets isolated from *Akita* mice, the mutant proinsulin inhibits insulin production from co-expressed wild-type proinsulin alleles ⁷². Further, species-specific or epitope-tagged versions of proinsulin allowed clear distinction between wild-type 'innocent proinsulin bystanders' and mutant gene products. Using a construct known as hProCpepGFP (human proinsulin with green fluorescent protein contained within the C-peptide), human insulin and CpepGFP are generated in the secretory granules of rodent pancreatic beta cells ⁷². Transgenic mice expressing hProCpepGFP in pancreatic beta cells are normal, but when mated to *Akita* mice to develop *MIDY*, the bystander hProCpepGFP precursor is blocked in the secretory pathway of islet beta cells; in parallel, production of the

CpepGFP product also becomes blocked⁷⁰. Interestingly, an hProCpepGFP bearing the C(A7)Y *MIDY* mutation inhibited the export of co-expressed wild-type proinsulin (resulting in high molecular weight protein complexes that included both mutant and nonmutant gene products), while an untagged version of mouse *Akita* protein inhibited wild-type human insulin production in pancreatic beta cells⁷². Initially, the trafficking blockade seems to involve specific recruitment of bystander proinsulin into aberrant disulfide-linked proinsulin-containing complexes while other secretory proteins are normally trafficked and secreted^{69, 72}. More work is needed to precisely characterize the aberrant disulfide-linked proinsulin-containing protein complexes, but recent studies suggest that improperly oxidized, high molecular weight species of wild-type proinsulin are recovered in increased amounts when the oxidizing environment of the ER is perturbed¹⁵. Alternatives that need to be tested (which are not mutually exclusive) are that proinsulin mutants causing *MIDY* might directly engage wild-type proinsulin in aberrant disulfide-linked protein complexes, or might indirectly recruit wild-type proinsulin into such complexes through changes in the oxidizing environment of the ER. Through either of these mechanisms, the expression of proinsulin mutants that cause *MIDY* blocks wild-type proinsulin in the ER, causing insulin deficiency.

Findings consistent with this basic paradigm are now being replicated with many of the newly-described *MIDY* mutants. For example, recent examinations of human *MIDY* mutants typically exploit species-specific antibodies and constructs, or distinct, epitope-tagged versions⁵⁸⁻⁶⁰. To greater or lesser degrees, each of these most recent studies supports the notion of dominant-negative inhibition of trafficking of co-expressed proinsulin, which occurs at a stage before beta cell death; indeed, demonstration of these bystander effects requires ongoing secretory protein synthesis.

In later stages of *MIDY*, as beta cell dysfunction in diabetic individuals worsens, other co-expressed secretory proteins may become blocked in the secretory pathway⁷³. Moreover, affected individuals convert from having normal beta cell mass to having a loss of beta cell mass⁷⁰. Indeed, the higher the expressed fraction of misfolded proinsulin, the more beta cell demise is accelerated, and the greater is the activation of ER stress responses that progress even further after diabetes onset⁶⁹. Among other things, chronic activation of ER stress response pathways leads to induction of the Chop protein that is strongly implicated in beta cell apoptosis^{74, 75}. While the present review has not focused specifically on the links between ER stress response pathways and beta cell death, we do not exclude the fact that cell death pathways are highly likely to participate in the natural progression of *MIDY*, as a final common pathway leading ultimately to a decrease in beta cell mass.

Conclusion

We are beginning to understand that *MIDY* links the earliest steps of proinsulin folding and misfolding to the cell biology of the secretory pathway in pancreatic beta cells. In *MIDY*, evidence indicates that dominant-negative diabetes is directly linked to the quantity of misfolded proinsulin expressed. Yet other genetic and epigenetic modifiers may contribute to *MIDY* phenotypes. Patients with several *MIDY* mutations, including proinsulin-A(SP24)D, G(B8)S, G(B8)R, C(B19)G, or R(Cpep-2)C, have a wide range of clinical severity even among family members bearing the same mutation — including the possibility of absence of diabetes throughout life. Developing therapeutics that may bring about such an outcome may have relevance far beyond *MIDY*. Specifically, the work described in this review raises new questions about beta cell failure in more common forms of type 2 diabetes, in which augmented absolute levels of misfolded proinsulin might bring about similar dominant inhibition of insulin production, ER stress, and beta cell demise.

Box 1**ER stress response pathways and proinsulin biosynthesis and misfolding**

1. PERK (PKR-like Endoplasmic Reticulum *Kinase*) is an ER signaling protein responsible for phosphorylating eIF2 α , and increased PERK activity facilitates the cellular response needed to coordinate the synthesis of proinsulin with its folding and trafficking^{25, 76–81}. Defects in PERK-mediated phosphorylation of eIF2 α result in increased proinsulin misfolding and ER retention caused by impaired ER homeostasis²⁹; importantly, these phenotypes occur even in the absence of any *INS* gene mutations.
2. Ire1 α is a conserved ER signaling protein that is also expressed in pancreatic beta cells. Knockdown of Ire1 α in a pancreatic beta cell line markedly decreases proinsulin biosynthesis³⁴, while chronic activation of Ire1 can also result in decreased proinsulin biosynthesis through preproinsulin mRNA turnover^{34, 82}.
3. ATF6 is the third ER membrane signaling component of the tripartite ER stress response. ATF6 overexpression inhibits insulin gene expression⁸³ and can trigger beta cell apoptosis, but its physiological overexpression is held in check by facilitated degradation involving the Wolfram syndrome gene product, WFS1⁸⁴.

Acknowledgments

This review was supported primarily by National Institutes of Health Grant R01 DK 48280 (to P.A.), and also to R00 DK077441A (to I.H.).

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Figure 1.

Proposed molecular mechanisms of beta cell failure caused by misfolded (pre)proinsulin. When WT (blue) and mutant (red) proinsulin are co-expressed in beta cells, the mutant proinsulin promotes WT proinsulin retention in the ER, decreasing insulin production, and forcing beta cells to use up insulin secretory granules from their progressively depleted insulin storage pool. The insufficiency of insulin increases blood glucose that initially stimulates even more production of wild-type and mutant preproinsulins, exacerbating ER stress induced by misfolded proinsulin-containing protein complexes. Inexorable progression of insulin insufficiency leads to frank diabetes, further ER stress, and beta cell demise.

Table 1

Sequence of preproinsulin and site of MIDY mutations

	MIDY mutations		Ages of onset	Original diagnosis	References
	Nomenclature by peptide chain	Nomenclature by preproinsulin a.a.			
Signal Peptide	R(SP6)C or H	R6C or H	15-65yr ¹	MODY ¹	50,56,60
	A(SP23)S	A23S	6 yr	Type 1	66
	A(SP24)D	A24D	most <6 mo ²	Neonatal DM ²	49,50,62
Insulin B Chain	H(B5)D	H29D	<6 mo	Neonatal DM	50
	L(B6)M, V or P	L30M, V or P	<6 mo or 17-38 yr ³	Neonatal DM or MODY ³	53,60
	G(B8)S or R	G32S or R	most <6 mo ⁴	Neonatal DM or Type 1 ⁴	49-50,54,66
	L(B11)P	L35P	<6 mo	Neonatal DM	50,53
	LY(B15,16)H	L39Y delinsH	<6 mo	Neonatal DM	53
	C(B19)G	C43G	most <6 mo ⁵	Neonatal DM ⁵	49-50
B-C junction	R(B22)Q	R46Q	17-24 yr	MODY	51,56
	G(B23)V	G47V	<6 mo	Neonatal DM	49-50
	F(B24)C	F48C	<6 mo	Neonatal DM	49-50
	R(Cpep-2)C	R55C	<6 mo or 8-37 yr	Type 1 or MODY	51,60
C-Peptide	G(Cpep28)R	G84R	<6 mo	Neonatal DM	50
C-A junction	R(Cpep+2)C	R89C	most <6 mo ⁶	Neonatal DM ⁶	49-54
Insulin A Chain	G(A1)C	G90C	<6 mo	Neonatal DM	49-50
	C(A6)Y	C95Y	<6 mo	Neonatal DM	53
	C(A7)Y or S	C36Y or S	<6 mo	Neonatal DM	49-50,62,55
	S(A12)C	S101C	<6 mo	Neonatal DM	50
	Y(A14)C	Y103C	<6 mo	Neonatal DM	14

MIDD mutations		Ages of onset	Original diagnosis	References
Nomenclature by peptide chain	Nomenclature by preproinsulin a.a.			
Y(A19)C or Stop	YIOSC or stop	<6 mo	Neonatal DM	49-50,53

¹ One carrier of R(SP6)H, age 20, did not have DM (56-60).

² Age of onset 5 wk - 7 y in 3 members of the same family (50)

³ Proband's mother, age 68, carries mutation without DM (60)

⁴ Age of onset 5 mo - 22 y in 4 members of the same family(50)

⁵ Proband's father developed diet-controlled type 2 DM beginning at age 30 (49)

⁶ Three family members developed DM age 2 - 4 y (51).

* The native SP23 of chimpanzee preproinsulin is Ser. In the cartoon on the right, the normal position of Cys residues is overlaid in pink; disulfide pairs shown with red lines

MIDD mutation sites overlaid in green.