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Islet Amyloid in Type 2 Diabetes, and the Toxic Oligomer Hypothesis

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Type 2 diabetes (T2DM) is characterized by insulin resistance, defective insulin secretion, loss of β-cell mass with increased β-cell apoptosis and islet amyloid. The islet amyloid is derived from islet amyloid polypeptide (IAPP, amylin), a protein co-expressed and cosecreted with insulin by pancreatic β-cells. In common with other amyloidogenic proteins, IAPP has the propensity to form membrane permeant toxic oligomers. Accumulating evidence suggests that these toxic oligomers, rather than the extracellular amyloid form of these proteins, are responsible for loss of neurons in neurodegenerative diseases. In this review we discuss emerging evidence to suggest that formation of intracellular IAPP oligomers may contribute to β-cell loss in T2DM. The accumulated evidence permits the amyloid hypothesis originally developed for neurodegenerative diseases to be reformulated as the toxic oligomer hypothesis. However, as in neurodegenerative diseases, it remains unclear exactly why amyloidogenic proteins form oligomers *in vivo*, what their exact structure is, and to what extent these oligomers play a primary or secondary role in the cytotoxicity in what are now often called unfolded protein diseases. (*Endocrine Reviews* 29: 303–316, 2008)

I. Introduction

TYPE 2 DIABETES (T2DM) is a loosely defined clinical syndrome that likely has a number of different causes. Risk factors for T2DM include a positive family history, aging, and a variety of causes of insulin resistance, most commonly obesity (Refs. 1–4 and references therein). Most individuals respond to insulin resistance by adaptively increasing β-cell mass and insulin secretion to maintain normal blood glucose concentrations (5–7). This is consistent with the response of other endocrine organs when chronically stimulated. As an example, the low ionized calcium concentration in chronic renal failure provokes adaptive hyperplasia of parathyroid glands, not PTH failure (8).

Therefore, in those individuals who develop T2DM, the deficient adaptive response to increased insulin demand is an abnormal response. As such, by definition, T2DM is primarily due to insulin resistance and impaired insulin secretion.

A. β-Cell mass and type 2 diabetes

The underlying cause of impaired insulin secretion in T2DM is unknown and likely has multiple origins in different individuals. A relative deficit in the number of β-cells (often collectively referred to as β-cell mass) appears to be an important contributory factor (6, 9, 10). In obese individuals with impaired fasting glucose, β-cell mass is approximately 50% less than that of healthy individuals (6). The relationship between fasting blood glucose and β-cell mass is curvilinear, with a wide range of β-cell mass in nondiabetic individuals but with a steep increase in blood glucose with each decrement in β-cell mass beyond 50% (11) (Fig. 1).

In adult pigs, a 65% decrease in β-cell mass led to diabetes with most of the metabolic characteristics of T2DM (fasting and postprandial hyperglycemia, impaired insulin secretion, postprandial hyperglucagonemia) (12). Also, a 50% partial pancreatectomy in dogs or humans causes impaired fasting glucose initially and often diabetes subsequently (13–16). Collectively, these data imply that the deficit in β-cell mass present in T2DM can be sufficient to induce diabetes, particularly in the setting of associated insulin resistance.

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Abbreviations: AβP, Amyloid β protein; ATF6, activating transcription factor 6; BiP, binding Ig protein; CHOP, C/EBP homologous protein/GADD153; ER, endoplasmic reticulum; hIAPP, human IAPP; IAPP, islet amyloid polypeptide; IDE, insulin degrading enzyme; IRE1α, inositol requiring 1α; PERK, protein kinase-like ER kinase; rIAPP, rodent IAPP; T2DM, type 2 diabetes; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling; UPR, unfolded protein response.

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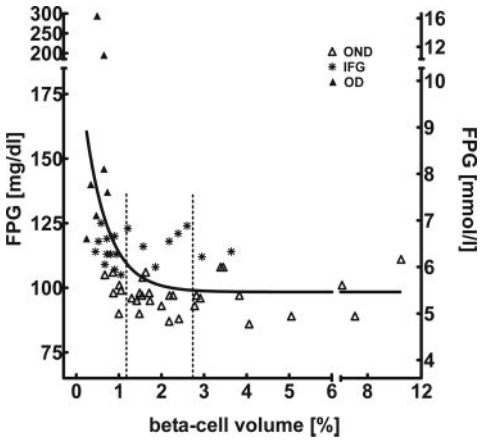

FIG. 1. Relationship between percentage of pancreas volume occupied by β-cells and fasting plasma glucose in obese humans without insulin or oral antidiabetic treatment. The *solid line* is derived from nonlinear regression analysis (monoexponential fit, $r = 0.50$; $P < 0.0001$ by ANOVA). The *dashed vertical lines* indicate the mean β-cell area in obese nondiabetic subjects (OND) (*right*) and the computed inflection point of the curve (*left*). IFG, Impaired fasting glucose; OD, obese diabetic. Adapted from Ref. 11. [Copyright 2006 American Diabetes Association. From *Diabetes Care* 29:717–718. Reprinted with permission from The American Diabetes Association.]

II. Islet Amyloid in T2DM and the Amyloid Hypothesis

The islet in T2DM is characterized by what was originally referred to as hyaline deposits, later demonstrated to consist of amyloid (17). The term amyloid was developed to describe abnormal extracellular deposits of protein that appeared somewhat like amylopectin (starch; amyloid "like amylopectin") (Fig. 2). Amyloid when present is always abnormal and consists of an insoluble protein precipitate, composed of protein monomers arranged in a β-pleated sheet structure (18). The resulting aggregated monomers appear as nonbranching fibrils by electron microscopy and are detected by congo red or thioflavine staining by light microscopy (Fig. 3). In 1987 two groups identified the constitutive protein in islet amyloid, naming it amylin and islet amyloid polypeptide (IAPP), respectively (19, 20). Because amylin also became the name of a pharmaceutical company, we have preferred IAPP. IAPP is coexpressed and cosecreted with insulin by pancreatic β-cells (21–23).

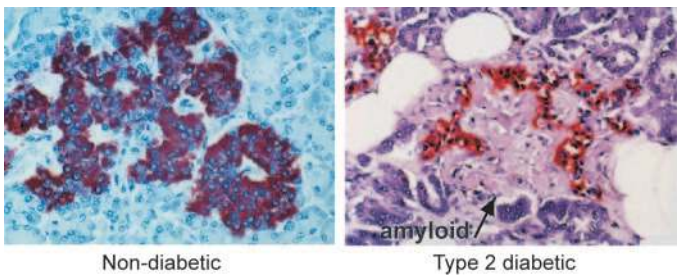

FIG. 2. Human islets from T2DM subjects (*right*) have less β-cells than those from nondiabetic subjects (*left*) and contain deposits of amyloid (*arrow*) derived from IAPP. Human islets were stained for insulin. This figure originally appeared in an article by Matveyenko and Butler (39). It is reprinted with permission from the *ILAR* Journal, Institute for Laboratory Animal Research, The National Academies (www.nationalacademies.org/ilar).

The islet in T2DM has parallels with the neuropathology in neurodegenerative diseases such as Alzheimer's disease (24–27), Parkinson's disease (28, 29), prion encephalopathy (30), amyotrophic lateral sclerosis (31), and Huntington's disease (32) (Table 1). In neurodegenerative diseases and T2DM, there is cell loss associated with abnormal aggregation of locally expressed protein. The proteins that form these aggregates share in common the propensity to form amyloid fibrils in an aqueous environment, prompting the question, is the formation of the amyloid fibrils in these diseases a cause or a consequence of the underlying cell attrition? Those in favor of a primary role of amyloid in neurodegenerative diseases coined the term "amyloid hypothesis" (24).

Evidence that was cited against the amyloid hypothesis is the observation that the severity of the disease state often correlates poorly with the extent of amyloid deposition (33–35). For example, the extent of dementia or cortical atrophy in Alzheimer's disease does not correlate well with the extent of brain amyloid derived from amyloid β protein (AβP) hypothesis (36). Islet amyloid is found in nondiabetic individuals, particularly with aging (37), and is not present in all islets in people with T2DM (38).

Evidence that supports the amyloid hypothesis includes the recapitulation of disease states by some (but not all) transgenic models for amyloidogenic proteins and mutations in amyloidogenic proteins that lead to early onset disease (26, 27, 39). Mutations in AβP that increase its propensity to aggregate were identified in early onset familial forms of Alzheimer's disease (25). A similar mutation in IAPP has been described in Japan and leads to an increased risk for T2DM (40).

This conflicting evidence for and against the amyloid hypothesis has been somewhat resolved by recent advances. The toxic form of amyloidogenic proteins appears not to be the extracellular amyloid fibrils detected by light microscopy, but rather smaller nonfibrillar oligomers (41–44). Before considering a revised model of the amyloid hypothesis further, we will briefly review the known properties of the protein that forms islet amyloid, IAPP.

A. IAPP physiological functions

IAPP is coexpressed with insulin by pancreatic β-cells (21–23). It is trafficked through the insulin secretory pathway and cosecreted with insulin, for example after meal ingestion. Although the physiological function of IAPP remains unknown, it is highly conserved between species, implying functional significance. Application of IAPP to rat soleus muscle strips was shown to inhibit insulin-mediated glucose uptake (45). The assumption was that the large amyloid deposits in the islet in T2DM would be associated with high circulating levels of IAPP, which in turn contributed to the insulin resistance of this disease. In reality, the circulating concentrations of IAPP (5–20 pM) were found to be far below that required to inhibit insulin action (in nanomoles), and furthermore plasma IAPP levels in T2DM were not increased compared with nondiabetic controls (21).

One well-characterized action of IAPP is a direct paracrine effect on β-cells to inhibit insulin secretion (46). It has also been suggested that IAPP delays gastric emptying and sup-

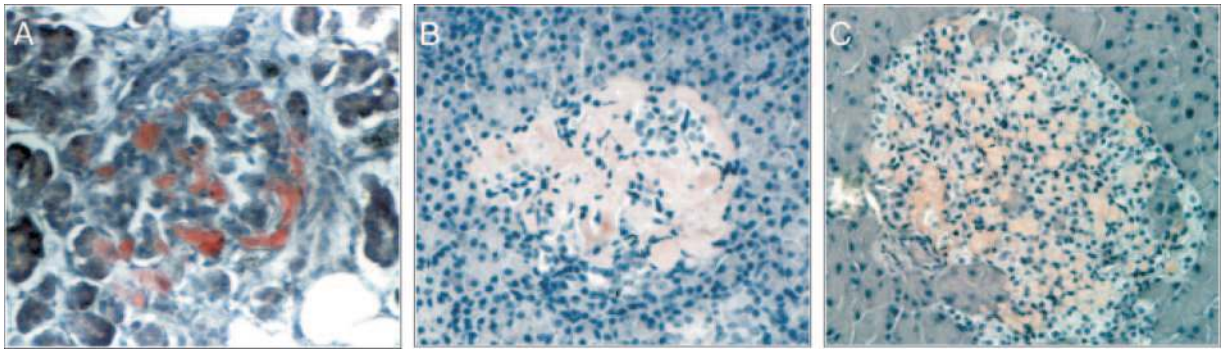

FIG. 3. The islets from T2DM human (A), diabetic vervet monkey (B), and diabetic obese hemizygous hIAPP transgenic mouse (C) stained for amyloid using Congo red. (Unpublished images from the Butler laboratory.)

presses appetite (47), although it is unclear whether these actions are physiological actions of IAPP within normal circulating levels or are present only in response to pharmacological IAPP concentrations.

B. IAPP species comparison

IAPP is expressed as an 89-amino acid protein that undergoes processing to a 37-amino acid peptide (48). The primary sequence of IAPP is closely conserved between species, but there are some important differences in $IAPP_{20-29}$, the region of the peptide believed to be important for conveying its propensity to form oligomers in an aqueous environment (49). As can be seen in Fig. 4, primates and humans share close homology in $IAPP_{20-29}$, and synthetic forms of these peptides form amyloid. It is therefore intriguing that nonhuman primates and cats, like humans, are also prone to developing T2DM with a similar clinical course and islet pathology as that observed in humans (50).

In contrast, neither rats nor mice spontaneously develop T2DM. Rat and mouse $IAPP_{20-29}$ is identical and not amyloidogenic, due to three proline residues that render rat and mouse IAPP water soluble (49). Moreover, the cytotoxicity of IAPP depends on its propensity to form oligomers (42, 51, 52). This distinction between human IAPP (hIAPP) and rodent IAPP (rIAPP) provides an opportunity to use the transgenic approach to examine the impact of hIAPP expression in mice or rats.

III. Lessons Learned from Transgenic Rodents; the Amyloid Hypothesis Challenged

Several different hIAPP transgenic mouse models have been reported (53–60). The phenotype and islet pathology of hIAPP transgenic rodents have been summarized in a recent review (39). Some but not all develop diabetes. In common with other mouse models of diabetes, there is a greater predisposition to diabetes in male mice compared with female mice, and also a background effect. hIAPP transgenic mice on a FVB background develop diabetes if IAPP expression is increased by induction of insulin resistance, whether through cross breeding onto an obese background (55, 58) or pharmacologically (53). Alternatively, increasing the gene dosage of hIAPP by cross breeding hIAPP transgenic mice to homozygosity also leads to diabetes (54). The underlying mechanism for diabetes in hIAPP transgenic mice and rats is a deficit in β-cell mass due to increased β-cell apoptosis (59, 61). The metabolic characteristics of T2DM, *i.e.*, hyperglycemia, impaired insulin secretion, insulin resistance, and hyperglucagonemia, are all recapitulated in hIAPP (HIP) transgenic rats (62).

Because replicating β-cells are particularly vulnerable to hIAPP-induced apoptosis (63), the deficit in β-cell mass is due to loss of cells as well as an inability to adequately compensate through increased β-cell replication. Transgenic protein expression in β-cells has unexpectedly induced diabetes in mice (64). Therefore, it is important to note that comparably high β-cell-specific transgenic expression of soluble rIAPP in mice does not lead to increased β-cell apoptosis, loss of β-cell mass, or diabetes (65). Collectively, these studies imply that hIAPP can be expressed and trafficked successfully by mouse β-cells up to a threshold beyond which apoptosis may be induced, and this vulnerability to high expression rates of hIAPP depends on its propensity to oligomerize.

An important lesson that arose from studies of hIAPP transgenic mice is that the concept that extracellular IAPP amyloid causes β-cell apoptosis (the amyloid hypothesis) is implausible. Homozygous transgenic mice for hIAPP developed diabetes due to a high rate of β-cell apoptosis by 10 wk of age (54, 65). However, extracellular islet amyloid was not yet present in these mice during the rapid loss of β-cells from

TABLE 1. The common molecular basis of amyloid-related T2DM and neurodegenerative diseases

Diseases	Protein that forms toxic oligomers	Cells lost
Type 2 diabetes mellitus	Islet amyloid polypeptide	β-cells
Alzheimer's disease	β-Amyloid protein	Cortical neurons
Parkinson's disease	Synuclein	Dopaminergic neurons
Prion encephalopathy/transmissible spongiform encephalopathies	Prion	Cortical neurons
Amyotrophic lateral sclerosis	Mutant superoxide dismutase	Motor neurons
Polyglutamine/Huntington's disease	Huntingtin's polyglutamine	Pyramidal neurons

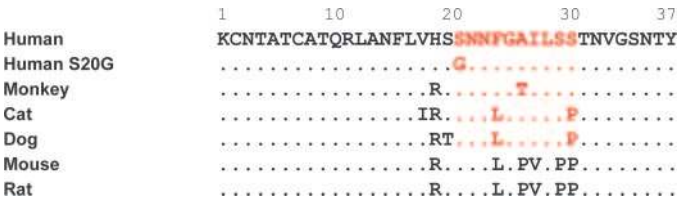

FIG. 4. Alignment of IAPP ortholog proteins. Amino acid alignment of IAPP protein sequences identified in *Homo sapiens* (human, CAA39504), human mutant (S20G) (40), *Macaca mulatto* (monkey, XP_001098290), *Felis catus* (cat, NP_001036803), *Canis lupus* (dog, NP_001003233), *Mus musculus* (mouse, NP_034621), and *Rattus norvegicus* (rat, NP_036718). *Dots* correspond to conserved residues with human IAPP sequence. *Red letters* correspond to the amyloidic sequence.

age 5–10 wk. In obese hemizygous hIAPP transgenic mice that develop diabetes at approximately 20 wk of age, extensive islet amyloid develops, but there is no relationship between the extent of islet amyloid and the frequency of β-cell apoptosis (61). Moreover, the β-cells undergoing apoptosis are not adjacent to amyloid deposits, as would be expected if the amyloid deposits were the toxic form of amyloid (Fig. 5). This lack of proximity of β-cells undergoing apoptosis and islet amyloid is also evident in humans with T2DM (6). Further evidence against the toxicity of extracellular amyloid is provided by another hIAPP transgenic mouse model that develops extensive islet amyloid but not diabetes (66).

Because cytotoxicity induced by hIAPP overexpression and amyloid formation were readily dissociated in hIAPP transgenic mice, the amyloid hypothesis in its literal form, that extracellular amyloid causes cytotoxicity, was challenged. The dissociation of hIAPP induced cytotoxicity and hIAPP amyloid formation gives rise to the question, what is the cytotoxic form of hIAPP?

A. hIAPP toxic oligomers, and not amyloid, induce β-cell apoptosis; the amyloid hypothesis modified

Cytoxicity by hIAPP was first reported when hIAPP or rIAPP was applied to human islet cells in culture (51). Application of hIAPP but not rIAPP caused β-cell apoptosis. Not surprisingly, the islet cells exposed to hIAPP (but nor rIAPP) were also subsequently observed to be decorated with amyloid. The authors concluded that the association between the appearance of extracellular amyloid fibrils and apoptosis implied causality, *i.e.*, that the extracellular amyloid fibrils had induced apoptosis. However, in subsequent studies other investigators were unable to support this conclusion (42, 52). When amyloid fibrils were added to islet cells in culture, apoptosis was not induced and electron microscopy revealed viable cells decorated with amyloid. In contrast, if a freshly prepared aqueous solution of hIAPP was added to islet cells in culture apoptosis was reproducibly induced, and under these circumstances electron microscopy revealed the presence of small nonfibrillar hIAPP oligomers, apparently disrupting the cell membrane, and indeed penetrating the cell (42). These data together with those from the hIAPP transgenic mice implied that it was not the amyloid fibrils that induce apoptosis, but much smaller oligomers that form rapidly after free hIAPP monomers interact in an aqueous environment.

This impression was reinforced by studies of membrane bilayers (42) (Fig. 6). Freshly prepared solutions of hIAPP induced nonselective ion channels and ultimately disrupted the membranes, whereas neither amyloid fibrils nor freshly prepared solutions of rIAPP had any discernible effect. Dissociation between formation and actions of hIAPP toxic oligomers *vs.* hIAPP-derived amyloid was further demonstrated by use of rifampicin (153). Rifampicin, as previously reported, inhibited hIAPP amyloid formation but failed to inhibit formation of either hIAPP toxic oligomers or hIAPP

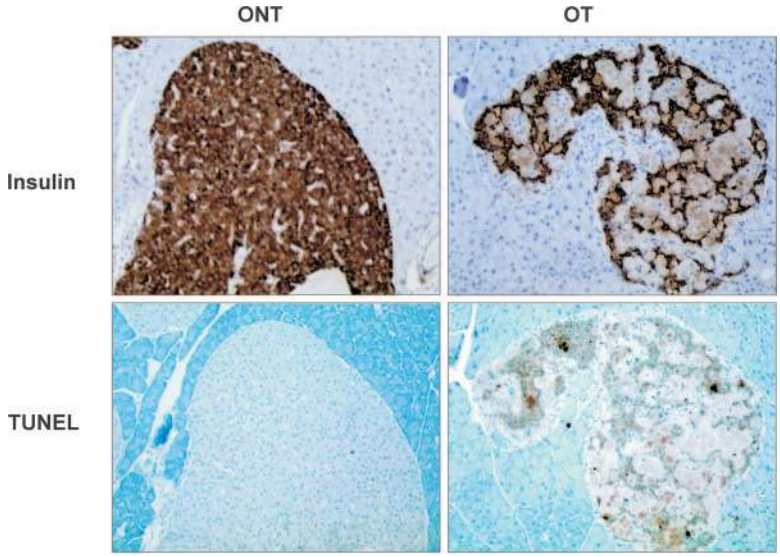

FIG. 5. The increased β-cell apoptosis in hemizygous hIAPP transgenic mice (OT) does not correspond to areas of amyloid. Islets from obese nontransgenic (ONT, *left panels*) and obese hemizygous hIAPP transgenic mice (OT, *right panels*) immunostained for insulin (*upper panels*) and corresponding islets stained for TUNEL (*lower panels*). Adapted from Ref. 61. [Copyright 2003 American Diabetes Association. From *Diabetes* 52:2304–2314. Reprinted with permission from The American Diabetes Association.]

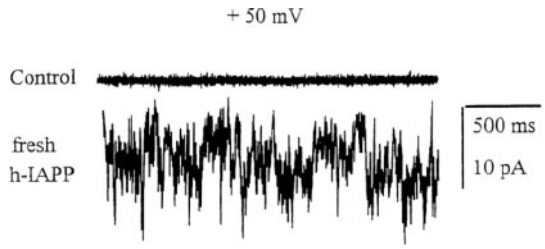

FIG. 6. Stability of planar bilayer membranes is disrupted by addition of hIAPP. Control recording of bilayer capacitance and the same membrane 5 min after adding 10 μmol/liter freshly dissolved hIAPP to the *cis* chamber. Note membrane instability and increase in membrane electrical noise. Filtered at 0.3 kHz. Adapted from Ref. 42. [Copyright 1999 American Diabetes Association. From *Diabetes* 48: 491–498. Reprinted with permission from The American Diabetes Association.]

cytotoxicity. These data established the fact that hIAPP toxic oligomers are not simply "pre-amyloid" fibrils or protofibrils, but are an off-amyloid fibril pathway form of oligomer (Fig. 7). This finding was already implicit in transgenic rodent studies where β-cell toxicity was unrelated to the extent or location of amyloid formation (61). If hIAPP toxic oligomer formation is off the fibril pathway, then inhibition of amyloid formation may not only fail to prevent toxicity of amyloidogenic proteins, but may even promote formation of toxic oligomers and enhance toxicity.

Having established that small membrane permeant oligomers, but not amyloid fibrils, are the toxic form of hIAPP, the amyloid hypothesis can be modified rather than rejected. Restated, proteins with the propensity to form amyloid fibrils have the capacity to form membrane-permeant toxic oligomers. To date, the relationship between the propensity to form toxic oligomers and amyloid fibrils is not fully characterized. Moreover, the structure of toxic oligomers remains to be established. Our own interest with respect to hIAPP toxic oligomers reverted to the questions, do toxic hIAPP oligomers form intra- or extracellularly, and *in vivo* do they act intra- or extracellularly to induce apoptosis?

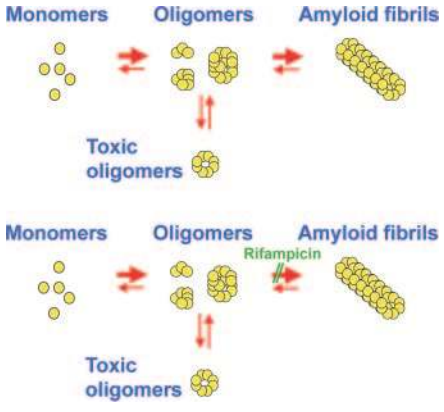

FIG. 7. Proposed model for the balance between the different aggregation states of hIAPP in an aqueous solution. Once hIAPP oligomers are dissolved in an aqueous solution, IAPP intermediate structures (protofibrils) further assemble into amyloid fibrils. Alternatively, they may form toxic membrane-perforating toxic oligomers (*top*). In the presence of rifampicin, formation of amyloid fibrils is inhibited, but the formation of toxic oligomers is unaffected, consistent with continued cytotoxicity (*bottom*). This figure originally appeared in an article by J. J. Meier *et al.*: *Am J Physiol Endocrinol Metab* 291: E1317–E1324, 2006 (153). It is used with permission from the American Physiological Society.

B. Where do hIAPP toxic oligomers form and act?

A recent breakthrough in this field came from the laboratory of Charles Glabe at the University of California, Irvine (68). Glabe's group developed a method to reproducibly synthesize a molecular mimic of AβP toxic oligomers using a colloidal gold core (69). When this molecular mimic was injected into rabbits, antibodies were raised that bound to the toxic form of AβP but did not bind to monomers or amyloid fibrils of AβP (68). Unexpectedly, this antibody also binds to the toxic oligomeric form of hIAPP, prion, and synuclein but not to the monomers or the amyloid fibrils of these proteins. One implication of this intriguing landmark paper is that the tertiary structure of the toxic oligomers of hIAPP, AβP, synuclein, and prion must be remarkably similar even though they are composed of monomers of distinct proteins. Another implication is that it is now possible to use this antibody to identify the location (intra- *vs.* extracellular) of the toxic oligomers. A third implication is that the mechanisms subserving formation of these toxic oligomers and their mechanism of action to induce cytotoxicity are likely to be similar in these diseases, now often referred to as unfolded protein diseases (70).

By use of the toxic oligomer-specific antibody, we were able to establish that the toxic oligomers of hIAPP in hIAPP transgenic mice form intracellularly (Fig. 8) (71). Staining for toxic oligomers was not found in the extracellular islet amyloid. To establish whether the toxic oligomers act intra- or extracellularly, we used a vaccine approach. After vaccination of hIAPP transgenic mice with the same oligomer preparation used by the Glabe laboratory to raise the antitoxic oligomer antibody, high titers of antioligomer antibodies developed in hIAPP transgenic mice. However, β-cell apoptosis was not decreased. By implication, toxic hIAPP oli-

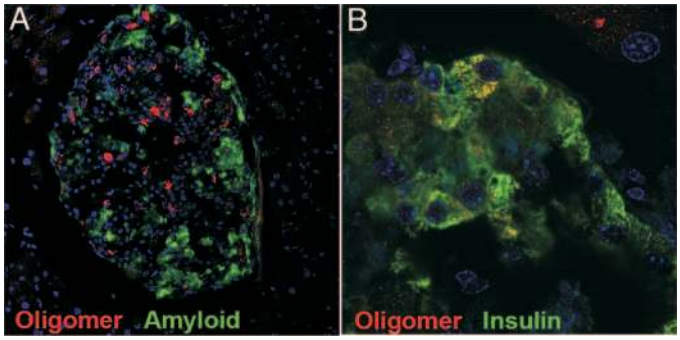

FIG. 8. hIAPP toxic oligomers in islets from obese hemizygous hIAPP transgenic mice. hIAPP toxic oligomer immunoreactivity does not coincide with extracellular amyloid; it is predominantly intracellular, confined to β-cells, and it is perinuclear or in vesicle-like structures. Immunofluorescent staining for toxic hIAPP oligomers (*red*), autofluorescence for amyloid (*green*), and nuclei 4′,6-diamidino-2-phenylindole (DAPI) (*blue*) (A, 20× magnification). Immunofluorescent staining for toxic hIAPP oligomers (*red*), insulin (*green*), and nuclei DAPI (*blue*) (B, 100× magnification). Adapted from Ref. 71. [Copyright 2007 American Diabetes Association. From *Diabetes* 56: 1324–1332. Reprinted with permission from The American Diabetes Association.]

gomers in hIAPP transgenic mice form and act intracellularly *in vivo*.

On a technical note, to date the antibodies for detection of toxic oligomers are ineffective in paraffin-embedded tissue, requiring frozen sections. This is not surprising because they detect the shared structure of toxic oligomers rather than a particular amino acid sequence in the monomers that compose them, a structure likely disturbed by tissue processing. Therefore, to date there are no data in human pancreas from patients with T2DM to confirm that the toxic hIAPP oligomers present in transgenic mice are also present in T2DM. Comparable intracellular IAPP oligomers were noted intracellularly in human β-cells from surgically resected insulinoma (72).

Although hIAPP oligomers form intracellularly in mice with high transgenic expression rates of hIAPP, not all β-cells even in these models have detectable hIAPP oligomers (71). These data imply that at any given time most β-cells in these mouse models have mechanisms in place that prevent hIAPP oligomer formation. Because hIAPP monomers rapidly form toxic oligomers in an aqueous solution, to better understand why these oligomers form in some β-cells, it seems prudent to consider the cellular mechanisms that prevent formation of hIAPP oligomers.

IV. Cellular Mechanisms to Prevent hIAPP Oligomer Formation. The Unfolded Protein Response (UPR), proIAPP Processing, and Vesicle Environment

The endoplasmic reticulum (ER) is responsible for the synthesis, folding, and appropriate targeting of all client secretory proteins before their export to the Golgi (most prominently insulin and IAPP in β-cells). The ER has several important properties to facilitate protein folding. These include a Ca^{2+} concentration of approximately 300 μM (*vs.* 0.1 μM in the cytosol) (73, 74), a relatively oxidative state favoring disulfide bond formation by protein sulfide isomerases (75), and a protein quality control system (76, 77). Unfolded proteins are exported from the ER by retrograde translocation to the cytosol and degradation by the proteosome (78, 79). In addition, the ER contains abundant chaperone proteins that shield hydrophobic regions of unfolded proteins from surrounding proteins (80–83). Chaperone protein binding has been shown *in vitro* to inhibit AβP oligomerization (84). Given that the ER protein concentration is approximately 100 g/liter, these properties are remarkably successful at preventing ER protein aggregation. This is particularly the case for proteins such as hIAPP that are highly prone to form self-aggregates at much lower concentrations in an aqueous environment (85).

In addition to these properties of the ER, the unfolded protein response (UPR) balances ER protein delivery with the capacity of the ER to fold and traffic these proteins [Refs. 86–90; also see article by Scheuner and Kaufman in this issue of *Endocrine Reviews* (152)] (Fig. 9). By doing so the UPR defends the ER from being overwhelmed by misfolded and more importantly aggregated proteins that may lead to ER stress and apoptosis (91). Three independent proteins, PERK (protein kinase-like ER kinase), IRE1α (inositol requiring 1α), and ATF6 (activating transcription factor 6) detect increased abundance of unfolded proteins in ER and activate a sequence of events that globally decreases translation of major ER client proteins, increases transcription and translation of ER chaperone proteins [*e.g.*, binding Ig protein (BiP)], and increases expression of proteins involved in clearance of unfolded ER proteins (88, 92, 93). The importance of PERK in the protection of β-cells was illustrated by the development of diabetes due to increased β-cell apoptosis in the PERK −/− mouse (94).

In summary, the UPR allows secretory cells such as the β-cell to balance ER delivery of major client proteins (insulin and IAPP in the β-cell) to the capacity of the ER to fold and traffic these proteins to the Golgi and secretory vesicles. Because expression of IAPP increases disproportionately to insulin under conditions of insulin demand (95), under these conditions, IAPP competes for ER resources and presumably constrains the maximal insulin synthesis rate. Theoretically, this can be overcome by increasing the number of β-cells. Although this adaptation appears readily available in mice (61), in adult humans there is a limited capacity for β-cell replication (6). Therefore, under conditions of sustained insulin resistance (*e.g.*, obesity), the ER in human β-cells will be in a state of prolonged high demand. Any additional stress to the β-cell under these conditions that adversely influences ER function (*e.g.*, oxidative damage, ER Ca^{2+} depletion, ER membrane leakage) will readily disturb this balance, potentially leading to ER stress-induced apoptosis (96, 97).

In common with the ER, the insulin secretory vesicles presumably sustain hIAPP concentrations that far exceed the solubility of hIAPP in a typical aqueous environment. A property of insulin secretory vesicles that is likely important in preventing IAPP oligomer formation is the acid pH of the vesicle lumen (85, 98, 99). At the pH 5.5 present in insulin secretory vesicles, hIAPP is maintained in monomers (99). In addition, insulin interacts with hIAPP to reduce oligomer formation (85). It is likely that there are other factors (chaperone proteins, ions) that restrain oligomer formation in insulin vesicles.

In conclusion, the synthesis and trafficking of nascent client secretory proteins by the ER is closely regulated to prevent oligomer formation. Likewise, the properties of the lumen of the insulin secretory vesicles favor maintaining hIAPP in a monomeric form. Because hIAPP oligomers apparently form intracellularly, these protective mechanisms presumably fail under those circumstances.

A. Why do hIAPP oligomers (and amyloid) form?

Given the potent mechanisms in place to prevent intracellular oligomerization of amyloidogenic proteins such as hIAPP, why does this fail in T2DM? Mice transgenic for hIAPP provide some insights. First, the increased risk of hIAPP oligomerization with increasing hIAPP expression implies that the mechanisms that protect against hIAPP aggregation and toxicity are saturable. This is consistent with the observation that circumstances that increase expression of IAPP per β-cell in humans increase risk for developing T2DM.

Thus insulin resistance (which disproportionately increases IAPP compared with insulin expression) (100, 101) is

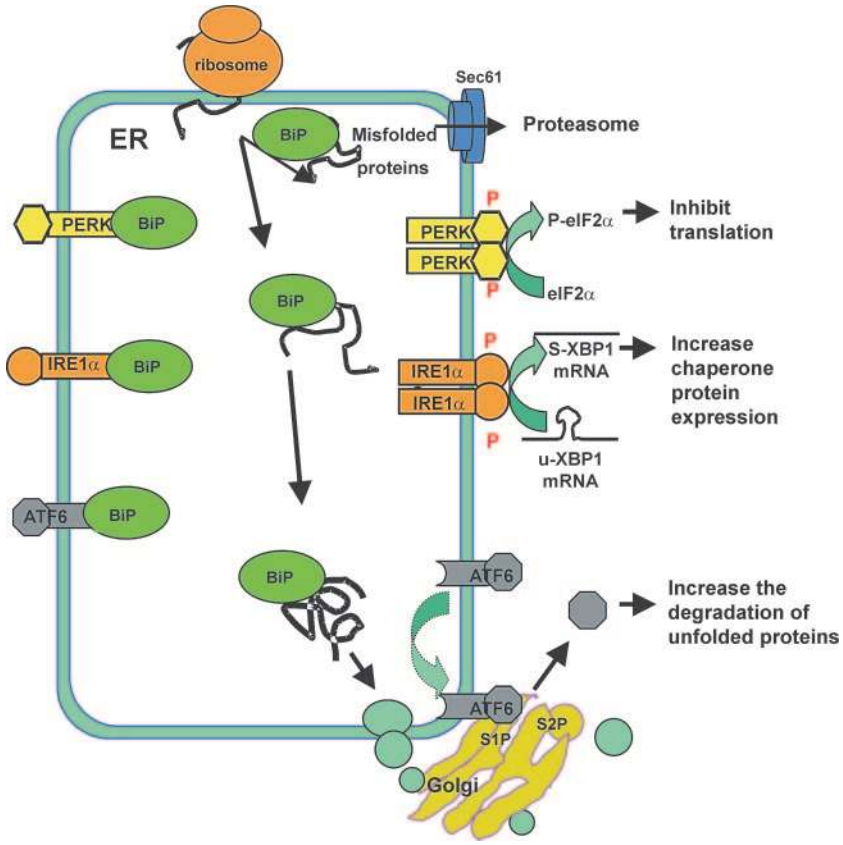

FIG. 9. The schematic illustration of the UPR in protein secretory cells. Increased demand for BiP leads to detachment of BiP from PERK, IRE1α, and ATF6, which get activated. Activated PERK phosphorylates α-subunit of eukaryotic translation initiation factor 2 (eIF2α), which subsequently suppresses ER protein translation and leads to more ATF4. Activated IRE1α has a RNA editing function and removes the hairpin structure on inactive X-box-binding protein-1 (XBP1) mRNA (unspliced, u-XBP1), which later becomes active transcription factor (spliced, s-XBP1). Activated ATF6 translocates into the Golgi and undergoes partial intramembrane proteolysis by site-1 protease (S1P) and site-2 protease (S2P), then migrates to the nucleus. These three activated transcriptional factors then induce a series of responses to increase chaperone proteins, limit new protein translation, and increase the degradation of unfolded proteins (see Fig. 10 and Refs. 65, 83, 86, 123, and 131).

a major risk factor for T2DM. Also, a 50% decrease in β-cell mass (which doubles the secretory demand per β-cell) often leads to subsequent diabetes in dogs (15) and humans (14, 16), both of which express an amyloidogenic form of IAPP, but not rats that secrete a soluble form of IAPP (102) (Fig. 4). Recent genome-wide linkage studies show linkage between risk for T2DM and several cell cycle transcriptional regulatory proteins (3). This together with the wide range of β-cell mass observed in nondiabetic humans (Fig. 1) raises the possibility that a relatively low adult β-cell mass might serve as a risk factor for T2DM. Under these circumstances, insulin resistance would place a substantial increased demand per β-cell in adult humans and, presumably, a greater risk for hIAPP expression rates that exceed the threshold for trafficking hIAPP in a soluble form.

Another potential cause of IAPP oligomer formation despite the protective mechanisms against it would be expression of a mutant hIAPP that increases the propensity for hIAPP to form oligomers (103). Although a rare cause of T2DM, the S20G mutation (Fig. 4) reported in Japan meets this criterion (40).

One other potential mechanism that would increase the risk for hIAPP toxic oligomer formation would be a decrease in the capacity of β-cells to neutralize toxic oligomers as they form. Insulin degrading enzyme (IDE) has been reported to have this property, and therefore it is of note that the IDE gene shows linkage to both T2DM and Alzheimer's disease (3, 104). IDE has been shown *in vitro* to inhibit hIAPP (and AβP) aggregate formation and cytotoxicity (105, 106). Polymorphisms in chaperone proteins important in trafficking hIAPP are an obvious candidate for increased propensity to form hIAPP oligomers. In this regard it is of interest that hIAPP and AβP share close structural properties and that the prevalence of Alzheimer's disease is increased in people with T2DM (107).

Also, any factors, inherited or acquired, that disturb the function of the ER might reasonably be expected to increase risk for hIAPP oligomer formation. Compromised ER function leads to mitochondrial dysfunction (108). Because ER function requires high energy, it is reasonable to expect that compromised mitochondrial function might lead to ER dysfunction. Therefore mitochondrial dysfunction in β-cells in T2DM (109) might be expected to lead to increased risk of hIAPP oligomer formation.

Factors in the secretory pathway and vesicle environment might also contribute to risk for oligomer formation. In cystic fibrosis, acidification of intracellular vesicles is impaired, and it is therefore of interest that a high proportion of patients

with cystic fibrosis develop T2DM with islet amyloid (110). Also, impaired hIAPP processing, a function of the insulin secretory pathway, has been implicated as a predisposing factor to hIAPP amyloid formation (111). This might be a factor in the tendency for islet amyloid formation in human insulinoma (72).

In conclusion, there are several potential factors already well recognized as associated with T2DM that might lead to an increased risk for hIAPP oligomerization. From the above discussion, it can be appreciated that the formation of toxic hIAPP oligomers might occur as a consequence or as a cause of β-cell failure. Because hIAPP oligomers are cytotoxic, it would seem reasonable to predict that they contribute to the progression of β-cell failure in T2DM, if they form secondary to islet dysfunction. Given the probable multiple causes of the clinical syndrome of T2DM, hIAPP oligomers likely form and contribute early in some forms of T2DM and late in others, depending on the underlying mechanisms that initiated β-cell dysfunction.

B. hIAPP-induced β-cell apoptosis

Apoptosis was first used to describe cell death with specific morphological characteristics (cell shrinkage, nuclear condensation, chromatin margination, and clumping and blebbing of the cell surface) (112) and then on the presence of free 3-OH strand breaks in DNA by the TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling) method (113). Subsequently specific biochemical pathways were identified that initiate and execute this form of cell death (114). Furthermore, apoptosis was found to be an evolutionarily conserved means of tissue remodeling during normal development (115). In adult tissue, apoptosis continues to play a role in regulated cell turnover (116). Induction of high rates of apoptosis has been identified as the underlying mechanism leading to loss of cell mass in neurodegenerative diseases, *e.g.*, Alzheimer's disease (117, 118) and Parkinson's disease (28).

Two major pathways of apoptosis are the extrinsic pathway (119) and the intrinsic pathway, which includes the ER stress pathway (91, 96, 97, 120–123). The extrinsic pathway is classically mediated by binding of death signals (Fas ligands) to death receptors (Fas) on the cell surface leading to aggregation of Fas receptors, caspase-8 and Fas-associated death domain protein into a death-inducing signaling complex in which caspase-8 is proteolytically activated and then released (119). Activated caspase-8 can then either directly activate the execution phase of apoptosis (via caspase-3) or amplify its signal by proteolytic activation of the proapoptotic member of the Bcl-2 family Bid leading to subsequent release of mitochondrial proapoptotic factors (*e.g.*, cytochrome c). The extrinsic pathway of apoptosis is active in autoimmune-mediated β-cell death (124, 125) and has also been invoked as a mediator of β-cell glucose toxicity (type-1 and type-2 diabetes) through the actions of high glucose concentration to induce expression of the Fas ligand and IL1-β in β-cells (126, 127).

The intrinsic pathway of apoptosis is mediated through a number of cell stresses. In the context of degenerative diseases, documented inducers of the intrinsic pathway include ER stress, mitochondrial dysfunction, generation of oxygen free radicals, metabolic toxins, disruption of the actin cytoskeleton, and anoxia (97, 128).

The ER stress pathway of apoptosis (Fig. 10) is a mechanism of apoptosis to which cells with a high secretory burden (such as β-cells) are particularly vulnerable (65, 91, 94, 123, 129–131). The primary defense mechanism against ER stress-induced apoptosis is the UPR (86–90). For clarity in this article, we define ER stress as the circumstances that provoke induction of apoptosis as a consequence of the accumulation of aggregated proteins. We distinguish this from the UPR that we define as the adaptive efforts by the cell described above to prevent ER stress. This is in distinction to some publications in this recently evolving field that use UPR and ER stress interchangeably. When the UPR is unable to clear the ER of unfolded, and especially aggregated proteins, ER stress by this definition may develop.

Two distinct circumstances can be anticipated that might lead to ER stress. In one, mutant proteins are synthesized with the property of an increased propensity to oligomerize. The common mutations of the cystic fibrosis gene (132), the Huntington's gene (133), some known mutations of the AβP gene (27, 29), and IAPP (40, 103) leading to familial Alzheimer's disease or T2DM have this property. The Akita mouse model of diabetes has a mutation in the insulin gene leading to ER aggregation of insulin, ER stress-induced β-cell apoptosis, and diabetes (123). In humans, a rare mutation in IAPP that increases the propensity of IAPP to oligomerize is linked to a familial form of T2DM (40, 103). The other more common circumstance is ER overload, the expression of an oligomeric protein such as hIAPP at a rate that exceeds the ER capacity to fold and traffic the protein. This fits with the known risk factors for T2DM including insulin resistance and a deficit in β-cell mass (for example after partial pancreatectomy) (13).

Marchetti *et al.* (134) showed increased markers of ER stress in isolated islets from patients with T2DM. Interestingly, β-cells showed modest signs of ER stress when the islets were cultured at normal glucose, but increased when the islets were cultured at higher glucose. This finding implies a genetic predisposition in islets from individuals with T2DM to ER stress when β-cells are chronically stimulated, a predisposition absent in islets of nondiabetic individuals. These data are consistent with the notion of a lower capacity to traffic and fold major client secretory proteins, such as IAPP in individuals with T2DM. We have previously reported that ER stress is characteristic of β-cells in humans with type 2 diabetes but interestingly not in type 1 diabetes (Fig. 11B) (65).

The exact mechanism linking protein oligomer formation and ER stress-induced apoptosis is unknown. One proposed mechanism is that toxic oligomers interact with the ER membrane leading to Ca^{2+} leakage (74, 135). This might directly lead to mitochondrial membrane permeability, leakage of cytochrome c, and activation of executioner caspases (caspase-3), as well as indirectly contributing to apoptosis by increasing the number of unfolded proteins in the ER due to depletion of ER Ca^{2+}. Ca^{2+} leakage from the ER can also activate ER-associated calpain, which can then directly induce apoptosis in a caspase-independent manner (136, 137).

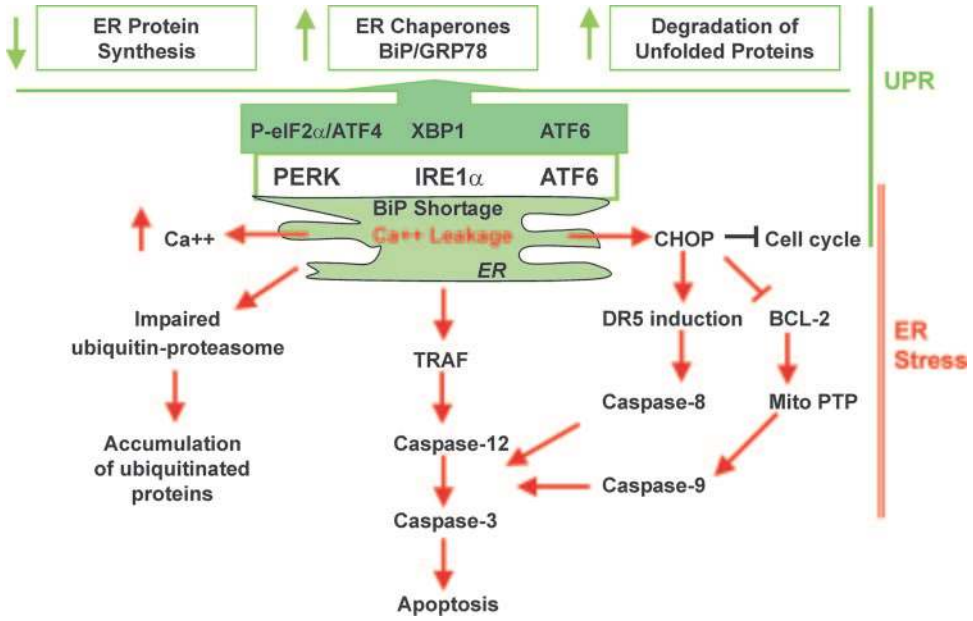

FIG. 10. Proposed molecular signaling pathways of hIAPP-induced ER stress and apoptosis in pancreatic β-cells. BiP shortage activates three transcriptional factors (ATF4, ATF6, and XBP1), which collectively launch the UPR. In hIAPP overexpressing models and under insulin resistance conditions, an increased number of proteins in the ER leads to molecular crowding, which promotes protein aggregation and misfolding, especially of the amyloidogenic protein like hIAPP. Aggregated or unfolded hIAPP can compromise ER membrane barriers for ionic calcium. Decreased calcium inside the ER lumen and increased calcium in the cytosol may lead to ER stress, which is represented by nuclear translocation of CHOP, induction of death receptor DR5, down-regulation of BCL-2, cleavage of caspase-12, and accumulation of ubiquitinated proteins. Decreased ER calcium will decrease the efficiency of protein folding machinery and result in more unfolded proteins. Increased calcium in the cytosol may open up the mitochondrial permeability transition pore (Mito PTP), leading to cytochrome c release and caspase-9 activation (64, 84, 87, 123, 131).

In rodents, activation of the ER membrane resident caspase-12 is associated with induction of the ER stress pathway of apoptosis, although it is not clear whether caspase-12 activation is a consequence of, or a mechanism contributing to the ER stress-induced pathway of apoptosis (31, 138, 139). Caspase-12 expression was detected in hIAPP transgenic mice and rats, but not in rIAPP transgenic mice (Fig. 11B). Caspase-4 appears to have the same properties (activated by chronic ER stress) in humans (117, 140).

ER stress has been identified as an important mechanism inducing apoptosis in Alzheimer's disease (117, 118, 141), Parkinson's disease (29, 142), and T2DM (65, 123, 131), all three of which share the characteristic of increased apoptosis in relation to protein misfolding of amyloidogenic proteins (Table 1). Therefore, ER stress is obviously a strong candidate for mediating hIAPP oligomer-induced apoptosis.

ER stress has been observed in β-cell lines transduced with hIAPP as well as mice and rats transgenic for hIAPP (65, 131) (Fig. 11A). Nuclear C/EBP homologous protein/GADD153 (CHOP) staining was found in hIAPP, but not rIAPP transgenic mice, and also in pancreatic section of a T2DM subject (Fig. 11B). The appearance of CHOP preceded execution of apoptosis as measured by TUNEL. We occasionally observed concordant nuclear and TUNEL in hIAPP-expressing INS cells (Fig. 11C). Furthermore, when we knocked down CHOP by small interfering RNA, apoptosis was decreased (Fig. 11D). Application of hIAPP oligomers extracellularly has also been shown to impair the ubiquitin proteasomal pathway (65). The accumulation of polyubiquitinated proteins was also identified in hIAPP, but not rIAPP transgenic mice (Fig. 11B). Because hIAPP oligomers induce membrane leakage and disruption, application of these oligomers extracellularly or formation of them within the secretory pathway intracellularly might reasonably be expected to permit Ca^{2+} influx into cytoplasm, a known signal to induce the intrinsic pathway of apoptosis. Moreover, ER stress has been shown to induce expression of death receptor (143), potentially invoking the extrinsic pathway of apoptosis so that in reality, both classical pathways of apoptosis will likely be active once cell membranes have been disrupted. Furthermore, a recent study showed that addition of hIAPP to the cells induces β-cell apoptosis through Fas-associated death receptor (144). Consistent with this, activation of p38 MAPK and JNK1 has also been noted after application of hIAPP oligomers to cells extracellularly (145–147).

Islet amyloid has also been noted in islets from hIAPP transgenic mice (56) and human islets either in culture at high glucose (148, 149) or after transplantation (150). It is not known why transplanted islets, or islets in culture, have increased hIAPP amyloid formation, but loss of vasculature might be a predisposing factor. Also, isolated islets and transplanted islets are relatively anoxic (151), with mitochondrial dysfunction likely leading to decreased ER function. hIAPP oligomer formation might be a contributory factor to early β-cell loss after islet transplantation.

V. Summary

The islet in T2DM shares much in common with neuropathology in neurodegenerative diseases such as Alzhei-

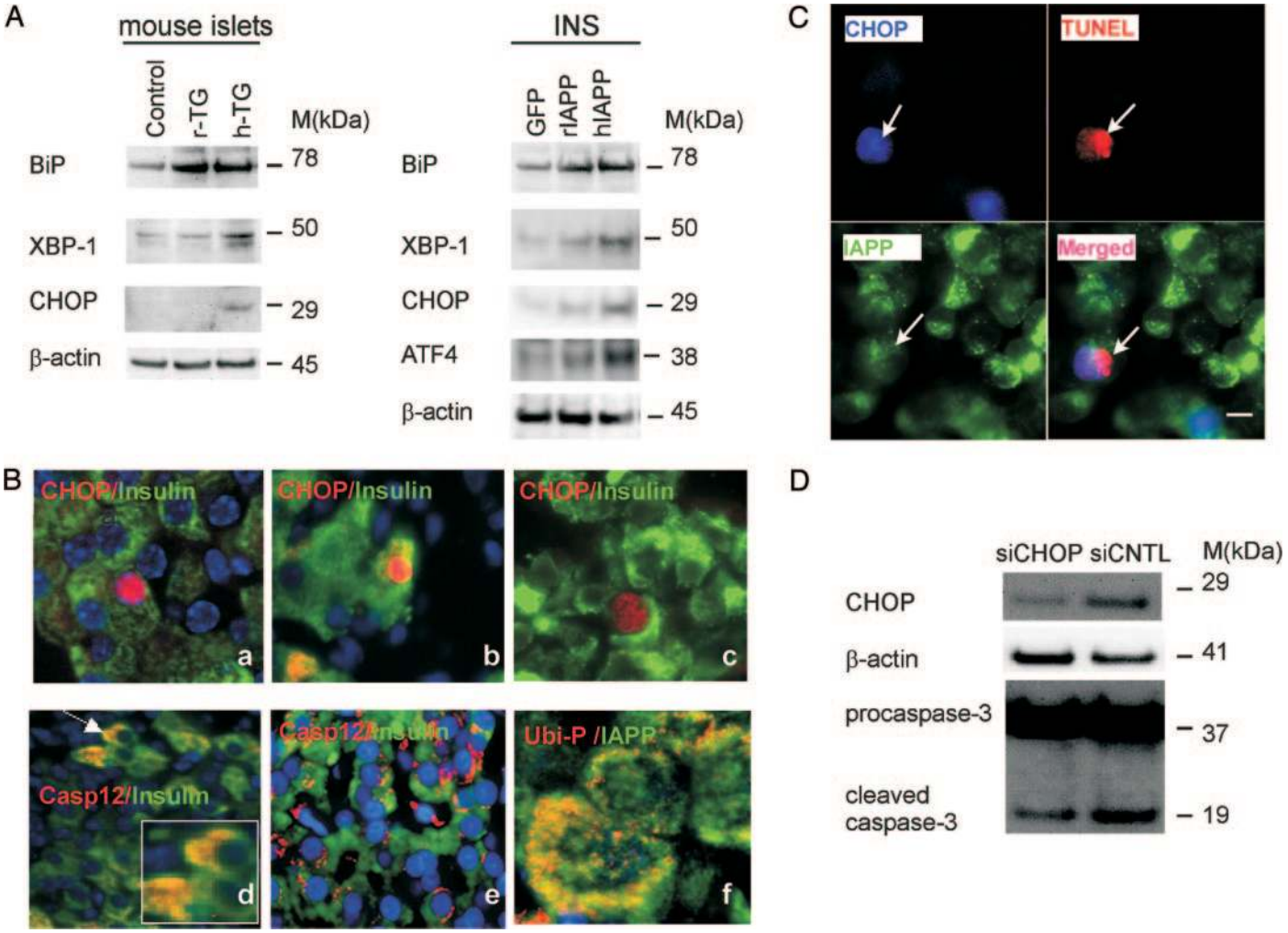

FIG. 11. hIAPP induces ER stress responses in pancreatic β-cells. A, Immunoblotting of UPR and ER stress markers in islets from wild-type, rIAPP and hIAPP transgenic mice (r-TG and h-TG, respectively), and INS cells overexpressing GFP, rIAPP-EGFP, or hIAPP-EGFP. B, Nuclear translocation of CHOP (a–c); increased caspase-12 expression (d, e), and accumulation of polyubiquitinated proteins (Ubi-P, f) in islets from hIAPP transgenic mouse (a, d, f), hIAPP transgenic rat (HIP rat; b, e), and human islets from an obese T2DM subject (f). C, Nuclear CHOP is colocalized with the appearance of TUNEL staining in INS cells overexpressing hIAPP-EGFP. D, Knockdown of CHOP by small interfering RNA reduces the cleavage of caspase-3 in INS cells overexpressing hIAPP. This figure is adapted from the original figure that appeared in an article by C. J. Huang *et al.* (65, 131). It is used with permission from The American Physiological Society. [Copyright 2007 American Diabetes Association. From *Diabetes* 56:2016–2027. Reprinted with permission from The American Diabetes Association.]

mer's disease. Because measurement of β-cell dysfunction is more sensitive and less demanding than cognitive function, hIAPP transgenic models are an appealing means to advance an understanding of both fields. Most of the focus in neurodegenerative diseases is now focused on protein misfolding and aggregation, the diseases now often referred to as unfolded protein diseases. Important questions that remain to be answered include, why do toxic oligomers of amyloidogenic proteins form; what is the precise structure of these oligomers; and are there therapeutic approaches that can prevent their formation or toxicity? Once these questions are addressed, the importance of the toxic oligomer hypothesis will be better defined.

Acknowledgments

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