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REVIEW

The receptor for advanced glycation endproducts is a mediator of toxicity by IAPP and other proteotoxic aggregates: Establishing and exploiting common ground for novel amyloidosis therapies

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Abstract: Proteotoxicity plays a key role in many devastating human disorders, including Alzheimer's, Huntington's and Parkinson's diseases; type 2 diabetes; systemic amyloidosis; and cardiac dysfunction, to name a few. The cellular mechanisms of proteotoxicity in these disorders have been the focus of considerable research, but their role in prevalent and morbid disorders, such as diabetes, is less appreciated. There is a large body of literature on the impact of glucotoxicity and lipotoxicity on insulin-producing pancreatic β-cells, and there is increasing recognition that proteotoxicty plays a key role. Pancreatic islet amyloidosis by the hormone IAPP, the production of advanced glycation endproducts (AGE), and insulin misprocessing into cytotoxic aggregates are all sources of β-cell proteotoxicity in diabetes. AGE, produced by the reaction of reducing sugars with proteins and lipids are ligands for the receptor for AGE (RAGE), as are the toxic pre-fibrillar aggregates of IAPP produced during amyloid formation. The mechanisms of amyloid formation by IAPP *in vivo* or *in vitro* are not well understood, and the cellular mechanisms of IAPP-induced β-cell death are not fully defined. Here, we review recent findings that illuminate the factors and mechanisms involved in β-cell proteotoxicity in diabetes. Together, these new insights have far-reaching implications for the establishment of unifying mechanisms by which pathological amyloidoses imbue their injurious effects *in vivo*.

Keywords: proteotoxicity; islet amyloid polypeptide; RAGE; pancreatic β-cells; diabetes; amyloidosis; aggregation

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Introduction

Proteotoxicity plays a role in many prevalent human disorders, including amyloidosis diseases. A range of human diseases is associated with amyloid formation and additional disorders are associated with proteotoxic aggregates that are not classified as classical amyloids. Amyloid formation refers to the

aggregation of normally soluble, functional proteins into insoluble fibrils that are partially ordered and β-sheet rich in structure. This process can occur in many different organs and tissues in the body, and plays a role in a broad range of over 40 different diseases, including Alzheimer's disease (AD), Parkinson's disease, and pancreatic islet amyloidosis; the latter being a major source of proteotoxicity to insulin-producing pancreatic β-cells in type 2 diabetes (T2D). An increasing number of studies on a variety of amyloidogenic peptides and proteins suggest that there are underlying commonalities in the mechanism(s) of amyloid formation and proteotoxicity, which are independent of the details of the polypeptide sequence. Consistent with the hypothesis that many amyloidogenic proteins invoke common mechanisms of cytotoxicity,[1] the receptor for advanced glycation endproducts (RAGE) engages multiple amyloidogenic proteins, such as islet amyloid polypeptide (IAPP or amylin), amyloid β (Aβ) peptide, serum amyloid A and prion-derived peptides.[2–6] However, of the amyloidogenic proteins that bind RAGE and activate RAGE-mediated pathological cellular signaling, only the specific kinetic species of IAPP that engage RAGE during amyloid formation have been meticulously characterized.[2] This provides a foundation and blue print for defining distinct toxic RAGE-binding entities in amyloidosis diseases, besides T2D, for which RAGE has been shown to impart pathogenic consequences.

T2D is characterized by the loss of β-cell function and mass, resulting in uncontrolled hyperglycemia and downstream complications. In T2D, β-cells are exposed to multiple diabetes-promoting factors, such as hyperglycemia (gluco- and glycotoxicity), hyperlipidemia (lipotoxicity), islet amyloidosis (proteotoxicity), inflammatory cytokines, and other factors for prolonged periods in subjects susceptible to metabolic disease. Each of these conditions triggers inflammatory perturbation in islet cells and other organs and, collectively, these considerations underscore the need for investigations focused on examining the impact of islet inflammation on β-cell fate and function. Pancreatic β-cells co-produce and co-secrete insulin and islet amyloid polypeptide (IAPP or amylin) in response to changes in blood glucose concentration. In the homeostatic state, IAPP acts both in the periphery and in the brain to regulate satiety, adiposity and metabolism. However, in T2D, IAPP undergoes amyloidosis in pancreatic islets by an unknown mechanism and self-assembles into proteotoxic oligomers that directly activate multiple cellular pathways leading to islet β-cell stress, dysfunction and death.[7–9] Recent work also demonstrates a role for human (h)IAPP in the cardiovascular complications of T2D,[10] and implicates a potential role for hIAPP in the pathogenesis of type 1 diabetes (T1D).[11] However, a lack of molecular understanding about the *in vivo* factors that promote pathological aggregation, and the upstream cellular mediators that regulate the downstream deleterious events in hIAPP-induced cytotoxicity has prevented identification of therapies to treat or prevent its pathologies. Here, we summarize the key biophysical and biochemical features, and cellular mechanisms by which pancreatic islet amyloidosis and other sources of β-cell proteotoxicity exacerbate diabetes; factors that are likely to play a role in the proteotoxicity of other amyloidogenic proteins.

Diabetes: A Growing Worldwide Crisis

Diabetes mellitus, commonly referred to as diabetes, describes a group of life-threatening metabolic disorders characterized by elevated blood glucose levels (hyperglycemia), resulting from the inability of the body to produce and/or utilize the essential hormone insulin, which is produced by pancreatic β-cells and regulates the transport of dietary glucose from the bloodstream into cells where the glucose is converted into energy. Individuals with diabetes have a higher risk of morbidity and mortality than the general population. Chronic hyperglycemia leads to the damage of various body organs and is the leading cause of disabling health complications, such as retinopathy, neuropathy, nephropathy, and cardiovascular disease. The worldwide prevalence of diabetes in adults has been steadily increasing over recent decades. According to the International Diabetes Federation (IDF), over 425 million people (one in eleven adults) worldwide are currently estimated to suffer from diabetes (not including 212 million who are undiagnosed), and the number is expected to grow to more than 640 million by 2045, representing slightly more than 10% of the world's adult population if current trends continue. The largest increases are expected to take place in the regions where society is transitioning from low-income to middle-income economies, where three quarters of people with diabetes live currently.[12,13] The human cost of diabetes is enormous. Diabetes is estimated to cause 5 million deaths per year among the 20 to 79-year-old age group, a number which exceeds the combined mortality from HIV/AIDS, tuberculosis and malaria.[12] The life expectancy of a diabetic patient is half that of an age-matched control subject in the United States.[2] The financial cost of diabetes is also enormous: 12% of global health expenditure is currently spent on diabetes ($727 billion), with over $673 billion worldwide in 2015 for T2D.[12,14]

The diagnosis and classification of diabetes are complex and have been debated and redefined over many decades. Diabetes can be diagnosed by measures such as blood tests that detect glycated hemoglobin (A1C), which indicate the average blood sugar level over the past two or three months, and/or by two or more separate blood sugar tests after

overnight fasting.[15] It is currently well accepted that there are many types of diabetes; three of these constitute the main forms: T1D, an autoimmune disorder whereby the body's immune system attacks insulin-producing pancreatic islet β-cells resulting in the inability to produce insulin and islet amyloid polypeptide (also known as IAPP or amylin); T2D, which develops as a result of inadequate insulin production and/or utilization (insulin resistance) over time due to various factors; and gestational diabetes mellitus, which is first detected during pregnancy.[12] Of the different forms of diabetes, T2D is the most common, accounting for 90% of diabetes cases.[12]

The Receptor for Advanced Glycation Endproducts (RAGE) and Its Ligands

Advanced glycation endproducts

The accumulation of advanced glycation endproducts (AGEs) in pancreatic islets, kidney, retina, nerves, and atherosclerotic plaques has been linked to diabetic complications.[2,16] AGEs are a heterogeneous repertoire of compounds formed by reducing sugars, such as glucose, that non-enzymatically modify amino acids, lipids, and nucleic acids, particularly during diabetes. In theory, every protein can be modified by glycation. A multitude of pathways lead to the formation of AGEs, which yield a large variety of products, including high molecular weight compounds and aggregated endproducts. First, a reversible Schiff base is formed by the reaction of the aldehyde group in the sugar with the ε-amino group of a lysine residue generating a double bond between the carbon atom of the sugar and the nitrogen in the lysine sidechain.[17] Subsequent Amadori rearrangement then leads to maturation of an AGE, or upon oxidation, may form glycoxidation products that facilitate the production of other distinct families of AGEs.[18] While AGEs come in many arrangements, there are three common outcomes following their formation: (1) Covalent cross-linking (particularly at basement membranes and on collagen), (2) disrupted osmotic balance of the system, and (3) receptor engagement by the best-characterized AGE receptor (RAGE) and other cell surface binding sites.[19] All of these may result in pathological consequences.[17]

AGE formation can induce aggregation of proteins into cytotoxic species.[20–28] The toxicity of glycated polypeptides may be due to the AGE modification or due to the conformational properties of the aggregated state. AGEs have also been demonstrated to play a mechanistic role in protein aggregation related to amyloidosis.[29–31] Glycation of albumin was first shown to promote the refolding of this globular protein from a largely α-helical to a cross-β structure commonly shared by all amyloids, demonstrating that glycation may be a predisposing factor for amyloid formation.[32] AGE-modified amyloid deposits have been found in the brain tissue of AD[33] and transmissible spongiform encephalopathy patients,[34] and in the pancreatic islets of diabetic patients.[35] High levels of argpyrimidine have been observed in protein aggregates derived from cases of familial amyloidosis but not in controls lacking disease, indicating an important potential role for methylglyoxal, an AGE precursor, in pathological protein aggregation.[36] Similar mechanisms have been reported in neurodegenerative disorders.[37–39] Studies have shown a correlation between AGEs and both the induction and progression of AD pathogenesis.[40–44] These data indicate that glycation of polypeptides, which have the propensity to aggregate into amyloid fibrils, can accelerate the formation of cross β-sheet structure.

AGE adducts and RAGE-mediated proteotoxicity

One of the main mechanisms by which both protein-AGE adducts and amyloidogenic proteins exert their action *in vivo* is via binding to multi-ligand cell surface receptors, such as RAGE, CD36, scavenger receptor class A and B type I, the serine protease tissue-type plasminogen activator receptor, and other pattern recognition receptors, which are expressed on a wide range of cell types.[45–52] Among the AGE binding receptors, RAGE is particularly important and well-characterized.[19,46,53–56] RAGE is an immunoglobulin-like, multimeric cell surface receptor with three distinct extracellular domains, including the variable (V)-type immunoglobulin (Ig) domain, which is primarily implicated in ligand engagement[19,57] [Fig. 1(A)]. RAGE is ubiquitously expressed on a variety of cell types including monocytes/macrophages, smooth muscle cells, endothelial cells, and pancreatic β-cells. Upon binding AGEs or one of its other ligands, including: IAPP, Aβ, prion peptides, transthyretin, S100/calgranulins, high mobility group box (HMGB) proteins, phosphatidylserine, and others, RAGE transduces signals through the intracellular formin molecule, Diaphanous 1 (DIAPH1).[2,58–61] A growing body of work in other cell types, particularly smooth muscle cells, cardiomyocytes and peripheral monocytes/macrophages, has revealed that DIAPH1 is required for RAGE-dependent upregulation of cytokines, oxidative stress, alterations in cellular migration and retention, proliferation and survivability, and other canonical facets of RAGE signaling[58,62,63] [Fig. 1(B)]. DIAPH1 is expressed in pancreatic β-cells. However, very little is known about the impact of DIAPH1 in these cells. Thus, further investigation of this pathway could potentially lead to a better understanding of the pathological consequences of RAGE engagement with its ligands in β-cells, particularly in diabetes. Of note, there are also other isoforms of RAGE, including soluble RAGE (sRAGE), which

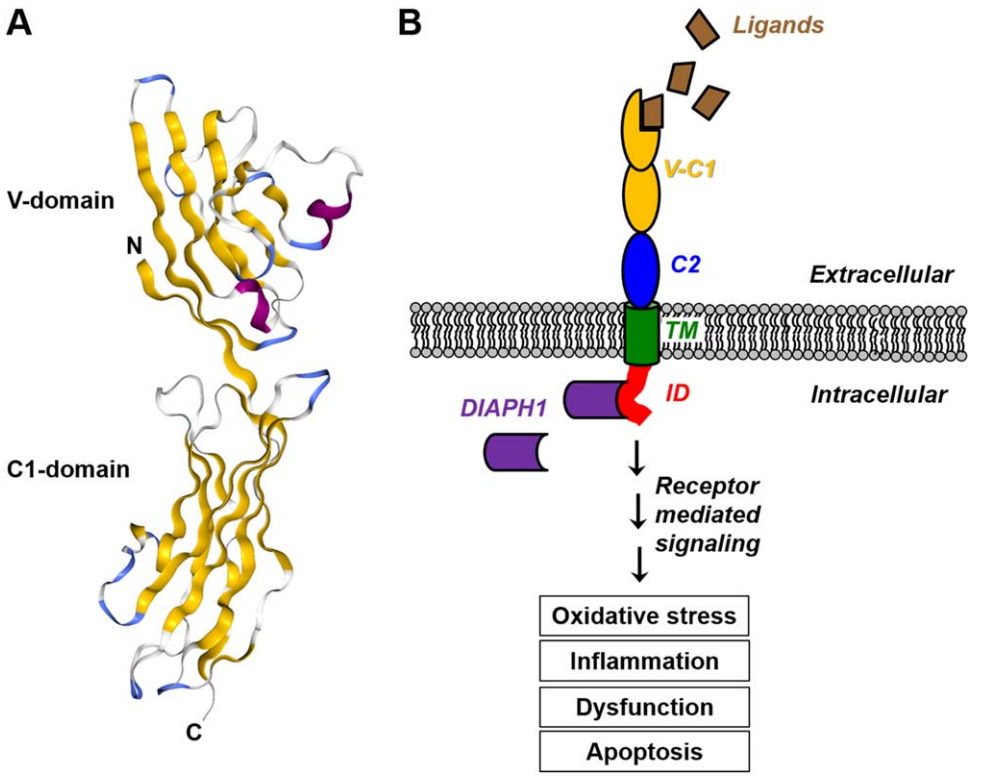

Figure 1. Schematic diagrams of extracellular RAGE structure and activation. RAGE is a cell surface receptor that is comprised of three extracellular immunoglobulin-like domains (a V-type domain and two C-type domains), a transmembrane domain (TM) and an intracellular domain (ID) that is required for signaling. (**A**) A ribbon diagram of the extracellular V-C1 domains of RAGE, which form an integrated structural unit. β-sheets are yellow, helices are purple and loops are blue and white (PDB code 303U).[54] (**B**) Activation of RAGE by its ligands leads to recruitment and binding of the intracellular formin molecule, DIAPH1, resulting in pathological intracellular signaling that can induce oxidative stress, inflammation, cellular dysfunction and apoptosis.

lacks the transmembrane and intracellular domains, and therefore fails to signal through DIAPH1 upon ligand engagement. sRAGE has been demonstrated to be protective as it acts as a decoy receptor that binds AGEs and other RAGE ligands, including some forms of amyloidogenic proteins, preventing their interaction with cell membrane-bound RAGE and the activation of pathological cellular signaling.[64] Together, the findings suggest that glycated proteins may constitute a family of amyloidogenic ligands with shared biophysical and biochemical properties that facilitate engagement and activation of pathological pattern recognition receptors. This hypothesis rationalizes how the V-domain of RAGE is capable of interacting with a diverse family of ligands. More work is needed to fully determine the specific protein structures and posttranslational modifications involved in the generation of toxic aggregates by AGEs.

Diabetes is among the diverse range of inflammatory diseases associated with RAGE activation

Increasing evidence implicates RAGE as an important mediating factor in diabetes and pancreatic β-cell dysfunction. A Genome Wide Association Study (GWAS) conducted on 3624 Finnish individuals revealed that the rs2070600 and rs17493811 *AGER (*AGER is the gene encoding RAGE) polymorphisms predicted increased risk of developing T1D.[65] rs2070600 has previously been shown to cause a decrease in sRAGE levels, and an increase in ligand affinity.[66–68] Other studies have also shown that RAGE induction and increased levels of RAGE ligands (S100B and other members of the S100/calgranulin family, HMGB1, and AGEs) drive dysfunctional glucose stimulated insulin secretion (GSIS),[69] amplify oxidative stress and impairment of adenosine triphosphate (ATP) synthesis, and increase apoptotic cell death in pancreatic β-cells.[70,71] In addition, RAGE inhibition *in vitro*, mediated by anti-RAGE antibodies or RNA silencing techniques, protected rodent and human β-cells from GSIS dysfunction and apoptosis.[72] Long-term treatment of cultured rat pancreatic islets with aminoguanidine, which inhibits AGE formation, conferred a benefit to β-cell insulin secretion and biosynthesis.[73] Subsequent studies have indicated that short term AGE exposure to rat pancreatic islets protected against apoptosis, whereas prolonged exposure promoted death. These findings suggest that the effect of RAGE activation may be dependent on the temporal dynamics of RAGE/ligand engagement and on ligand

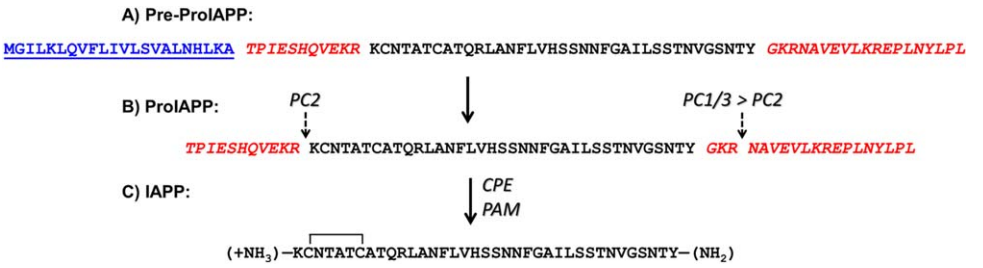

Figure 2. Processing of human pre-proIAPP into the mature IAPP sequence. (**A**) The 89 amino acid residues of pre-pro-peptide, including the 22-residue signal sequence which is shown in blue underlined font, and the flanking peptide regions of the pro-hormone, which are illustrated in red italicized font. (**B**) The sequence of proIAPP showing the cleavage sites for PC2 at the N-terminus of mature IAPP and PC1/3 after three residues (GKR) in the C-terminal flanking region. (**C**) Amidation of the C-terminal Tyr is a multi-step process. CPE removes the KR pair of dibasic residues that remain after PC1/3 cleavage, and the glycine is the nitrogen donor for amidation by PAM

concentration.[74] AGEs have also been implicated in destabilizing PDX-1 protein, a critical factor for β-cell survival, thereby impairing insulin synthesis.[75] Recent breakthroughs in the transplantation of human fetal pancreatic progenitor cells have shown promise in preventing RAGE upregulation in the kidney, protecting against diabetic nephropathy.[76] While these advancements are encouraging, more work is needed to fully determine how RAGE and its ligands influence pancreatic β-cell fate, and how modulation of ligand production, engagement of the ligand with the RAGE receptor, RAGE receptor, and DIAPH1-dependent intracellular processes may be harnessed to prevent β-cell dysfunction, diabetes induction, and subsequent pathological consequences.

Islet Amyloid Formation Is a Significant Source of β-Cell Proteotoxicity in T2D

The biosynthesis and processing of IAPP

IAPP belongs to the calcitonin peptide family, which is comprised of α- and β-calcitonin gene-related peptide (CGRP), calcitonin, intermedin, and adrenomedullin. These peptides all include an intramolecular disulfide bridge near the N-terminus and an amidated aromatic residue at the C-terminus.[77] IAPP has been found in all mammals studied and, like many polypeptide hormones, is synthesized as a pre-pro-form. In the case of the human polypeptide, the pre-pro-hormone is 89 residues in length and includes a 22 amino acid residue signal sequence at the N-terminus. The remaining 67 residues make up the pro-form (proIAPP), which includes N- and C-terminal extensions relative to mature IAPP (Fig. 2). ProIAPP is processed in the Golgi and in the insulin β-cell secretory granule to yield the mature hormone, which is secreted in tandem with insulin.[78–80] The N- and C-terminal flanking sequences of the pro-form are cleaved by the prohormone convertases, PC2 and PC1/3.[80] PC2 is responsible for cleavage of the N-terminal extension and PC1/3 is primarily responsible for the initial step in the C-terminal processing, although PC2 can cleave here as well. Processing at the C-terminus, which leads to an amidated C-terminal Tyr, is a multi-step process. The initial C-terminal cleavage leaves a Gly-Lys-Arg sequence as the new C-terminus. The Lys-Arg dipeptide is trimmed by carboxypeptidase, leaving the Gly, which donates the nitrogen during amidation by the peptidyl amidating monooxygenase complex (PAM).[79,80]

Defective post-translational processing of proIAPP may be another factor that promotes islet amyloid formation and proteotoxicity in T2D. There is indirect evidence that normal processing of proIAPP, at the N-terminal cleavage site, may be incorrect/incomplete in T2D, resulting in secretion of an N-terminal extended proIAPP intermediate.[77,81–83] Comparatively little work has been done on amyloid formation by partially processed proIAPP, but it has been proposed that retention of the N-terminal pro-sequence leads to binding to sulfated proteoglycans in the extracellular matrix, which in turn, could generate a high local concentration of the polypeptide and facilitate the formation of active amyloidogenic "seeds."[82,84,85] *In vitro* biophysical studies demonstrate that this partially processed form can bind to the glucosaminoglycan portion of proteoglycans, and that this interaction promotes amyloid formation.[82,84] Furthermore, the deposits formed can seed amyloid formation by mature fully processed IAPP.[84] Fully processed IAPP is stored in the halo region of the insulin secretory granule, while insulin is found in the dense core of the granule.[77,78] The concentration of IAPP in the granule is only about 1–2% that of insulin, but this is a much higher concentration than required to promote aggressive amyloid formation *in vitro*.[77,86–89] This suggests that there are factors that inhibit irreversible aggregation of IAPP in the granule.

Variations in the Primary Sequence of IAPP Correlate with its Ability to Form Amyloid *In Vitro* and *In Vivo*

IAPP is produced by all species examined to date, but not all IAPP sequences form amyloid. The

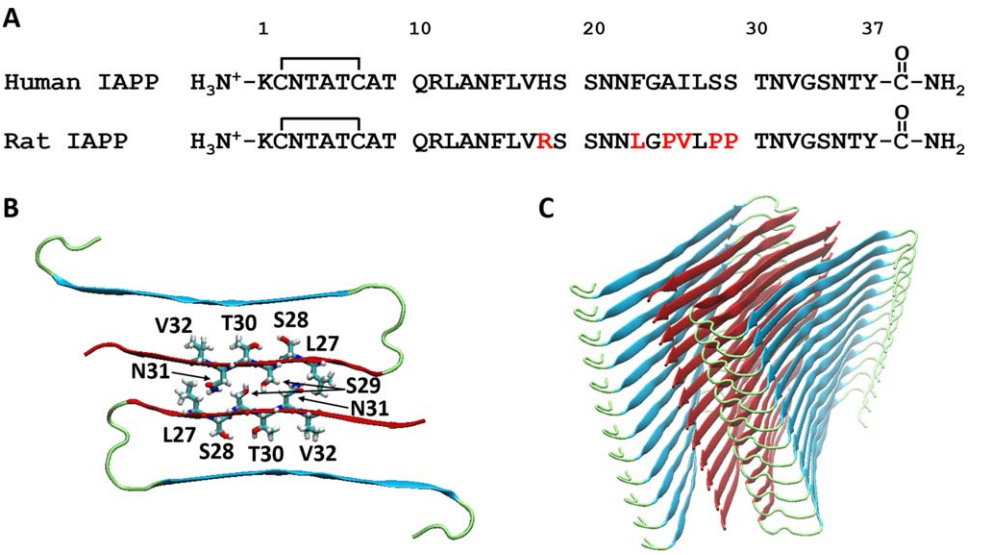

Figure 3. (**A**) The primary sequence of human and rat/mouse IAPP. (**B**) A cross section of the UCLA model of hIAPP amyloid fibril (104). Two symmetric related peptides are shown in a top-down view of a stack. Several residues which have been proposed to make key inter-stack contacts via sidechain interactions are indicated. (**C**) A ribbon diagram showing the arrangement of the polypeptide orientation in the amyloid fibril

sequence of IAPP is strongly conserved, however examined interspecies variations correlate significantly with the ability to form amyloid *in vitro* and with the presence or absence of islet amyloid *in vivo*. In particular, rat IAPP (rIAPP), which has the same amino acid sequence as mouse IAPP, differs from the human polypeptide at six positions and does not form islet amyloid *in vivo*. While there are some variations in the IAPP sequences of rodents, rodent IAPP generally is non-amyloidogengic *in vitro* under conditions where the human peptide rapidly forms amyloid (Fig. 3). Early comparison of the rodent and human sequences led to the hypothesis that differences in the sequence of IAPP within positions 20–29 correlated with the ability to form amyloid.[14] Four of the six sequence differences between hIAPP and rIAPP are found within this region (Fig. 3). The most important of these are three proline residues in rIAPP at positions 25, 28, and 29. Proline disrupts interstrand hydrogen bonding and intermolecular β-sheet formation, and this helps to explain the lack of islet amyloid in rodents. Another important difference between hIAPP and rIAPP is the replacement of His-18 in hIAPP with Arg in rIAPP. This substitution ensures that the sidechain at position-18 will be positively charged at all physiologically relevant pH values; the increased charge decreases the polypeptide's tendency to aggregate and form amyloid.[90,91] Naturally occurring sequence variations in IAPP have been reviewed recently.[86] Studies with synthetic variants of hIAPP have shown that residues outside of the 20–29 segment play an important role in modulating amyloidogenicity. For example, substitution of Asn-14 or Asn-21 can have drastic effects, as can proline substitutions outside the 20–29 region.[92,93] Conversely, replacement of residues Arg-18, Leu-23, and Val-26 in rIAPP by the corresponding amino acids of hIAPP (His-18, Phe-23, Ile-26) has been reported to lead to a weakly amyloidogenic polypeptide, despite it still containing the three proline substitutions found in rIAPP.[94] Taken together, these studies and other work demonstrate that additional factors other than just the sequence within residues 20–29 play an important role in dictating amyloidogenicity. Elucidating the factors that control hIAPP amyloidosis is important since it can aid in the rational design of next generation soluble analogs of hIAPP for clinical usage in the maintenance of metabolic homeostasis.[95]

The structure of monomeric hIAPP and of IAPP amyloid fibrils

Mature hIAPP is a positively charged, hydrophobic peptide that contains no negative charges at physiologically relevant pH's due to the lack of acidic residues and the amidated C-terminus. hIAPP does not adopt a well-defined globular conformation in its unaggregated state, but samples an ensemble of rapidly interconverting, partial and less structured conformations.[96] Thus, IAPP is considered to be a so-called "natively unfolded" or "intrinsically disordered" monomer, although residues 5 through 22 of hIAPP transiently sample helical phi psi angles in solution.[96] Interactions with membranes, particularly ones with a high content of anionic lipids, can promote formation of a more ordered partial helical state.[97,98] The tendency to form helical conformations may facilitate the conversion of hIAPP to β-sheet rich amyloid fibrils by promoting initial oligomerization.[99–102]

Several high resolution structural models have been proposed for hIAPP amyloid fibrils formed *in vitro* and, although they differ in the details, they share common overall features.[103,104] The basic fibril structure is made of two U-shaped stacks of IAPP monomers with two β-strands per monomer connected by a less ordered loop/turn region (Fig. 3). Each monomer is hydrogen bonded to its immediate neighbors in the same stack and the polypeptide forms parallel β-sheets. The backbone hydrogen bonds are between different molecules rather than within a single polypeptide, and are thus oriented parallel to the long axis of the fibril. The pair of U-shaped stacks are aligned so that hIAPP molecules in adjacent stacks are oriented antiparallel to each other, and the stacks interact via networks of side chain interactions. The different models vary in the exact location of the two β-strands and the loop/turn region. Both models place the disulfide-bridged loop between residues 2 and 7, outside of the ordered core of the cross β-structure (Fig. 3).

The physiological role of IAPP

The circulating concentration of IAPP is reported to be on the order of three to five picomolar in rats, and to rise to 15 to 20 picomolar with elevation of blood glucose levels.[77] The local concentration at the site of release from the granule is significantly higher and this is likely the more relevant value for amyloid formation. hIAPP is believed to play a role in controlling gastric emptying, maintaining glucose homeostasis, and suppressing glucagon release. The hormone is also involved in controlling satiety and is proposed to act as an adiposity signal.[105,106] A reduction in weight induced by IAPP has been reported for obese rats and humans, and animal studies have led to the hypothesis that weight loss occurs through a mode of action that is similar to that found in cases of enhanced leptin sensitivity.[107,108] As the focus of this review is on proteotoxicity rather than normal function of IAPP, the interested reader is referred to several recent reviews that discuss the proposed function(s) of IAPP in more depth.[77,86,89]

Does islet amyloid have an extracellular or intracellular origin?

In human T2D and in all cell and animal models, IAPP fibrils are found in invaginations of the β-cell membrane, but the question of where islet amyloid originates is still open to debate and there are conflicting reports in the literature concerning the initiation site for islet amyloid deposition *in vivo*. As noted, amyloid deposits found in human T2D appear to be extracellular and early studies with transgenic (Tg) mice were consistent with an extracellular origin for islet amyloid; but other studies with rodent models that overexpress IAPP are consistent with an intracellular origin.[77,109] It should be noted that some Tg mouse models of islet amyloidosis contain high copy numbers of the human IAPP gene and produce high levels of hIAPP. This could play a role in the reported intracellular aggregation. Defining whether islet amyloid has an intracellular or extracellular origin might affect drug design, and is thus of practical, as well as academic interest. The interested reader is referred to the recent review by Clark and co-workers for a more in-depth discussion of the issue of intra- vs. extracellular islet amyloid formation.[89]

Mechanisms of hIAPP-induced toxicity

Irrespective of the intra- and/or extracellular origin of IAPP aggregation, it is certain that discrete toxic forms of this polypeptide cause pathological consequences in key metabolic tissues. Indeed, identification of the toxic species in amyloidosis is a major challenge and the nature of the toxic species is controversial, due to the difficulty of isolating pure fractions of transient kinetic species that form over the course of amyloid formation. The preponderance of evidence indicates that soluble oligomeric species populated during amyloid formation are responsible for toxicity in islet amyloidosis and recent *in vitro* time-resolved biophysical and biological measurements have demonstrated that pre-amyloid oligomers are more toxic than hIAPP amyloid fibrils or monomeric hIAPP.[89,109,110] The biophysical properties of toxic hIAPP oligomers have been shown to be distinct from those produced in other amyloidosis diseases,[110] providing a target for rational drug development. However, the origin of the events that lead to β-cell death *in vivo* are still debated. Does induction of stress and dysfunction arise from within the cell or is it triggered from events that occur after secretion of the polypeptide from the β-cell? It has been proposed that hIAPP toxic oligomers are present in the cytoplasm of cells which overexpress hIAPP, and reactivity to an anti-oligomer antibody has been reported for β-cells in Tg hIAPP mice.[111,112] However, some of these studies made use of a conformational antibody, which was not raised against hIAPP. These antibodies are clearly useful *in vitro*, but their applicability to IAPP detection *in vivo* has been questioned.[113,114] Conversely, work with a cultured islet model that produces physiologically relevant levels of hIAPP show that, in this system, the secretion of IAPP is an important factor for islet amyloid formation and β-cell toxicity. In that study, inhibiting IAPP secretion, while maintaining the level of IAPP production, was found to reduce amyloid formation. In contrast, promoting increased secretion in this model without increasing hIAPP production led to increased toxicity and amyloid formation.[115] The differences observed between the various models may be related to the level of production of hIAPP.[89,114]

The pathways that lead to IAPP-induced β-cell dysfunction and apoptosis are not yet fully defined.[9,116–121] There are likely to be multiple mechanisms of toxicity and a variety have been reported; their relative contributions could depend upon cellular conditions. Defects in autophagy, increased production of pro-inflammatory cytokines, endoplasmic reticulum (ER) stress, permeabilization of cell membranes, mitochondrial membrane damage, activation of Calpain-2, receptor-mediated mechanisms including Fas and RAGE activation linked to induction of cell stress and apoptotic signaling pathways, have all been proposed to contribute to IAPP-induced β-cell cytotoxicity.[2,7–9,111,116–132]

Impairment of autophagy, and ER stress in hIAPP-induced β-cell proteotoxicity. Defects in autophagy play a role in the toxicity of a range of amyloidogenic proteins. In neurodegenerative diseases, for example, upregulation of autophagy acts as a protective adaptation to the accumulation of toxic amyloidogenic aggregates. However, lysosomal degradation of amyloidogenic polypeptides and autophagocytosis may not always be completely successful. The resulting accumulation of amyloidogenic aggregates can lead to autophagy-mediated lysosomal cell death or dysfunction. Along these lines, the overexpression of hIAPP in β-cells has been reported to lead to impairment in autophagy.[125,129,130] Stimulation of autophagy has been shown to protect against IAPP toxicity, while inhibition of autophagy-lysosomal degradation has been shown to enhance hIAPP-induced β-cell apoptosis.[125,130,133–135] ER stress, defects in the unfolded protein response (UPR) and in ER-associated protein degradation have all been proposed to contribute to hIAPP-induced β-cell death in T2D. ProIAPP and partially processed proIAPP could contribute to toxicity in cases where toxicity arises from intracellular aggregates since proIAPP mis-processing occurs in T2D, and processing is completed in the Golgi and insulin secretory granules.[77,86] It is important to note, however, that the role of ER stress in hIAPP-mediated toxicity is still controversial. Studies using exogenously added hIAPP and Tg mice that overexpress hIAPP have reported that ER stress is a mechanism of hIAPP-induced β-cell dysfunction,[111,124] while ER stress was not detected in studies employing cultured islets that produce more physiologically relevant levels of IAPP.[127]

Membrane disruption by hIAPP aggregates. Perturbation of membrane integrity by hIAPP has also been proposed to contribute to toxicity[89,128,132] and there is a large literature on *in vitro* studies of the ability of hIAPP to disrupt model membranes.[89,136] Unfortunately, it is not clear if there is a direct one-to-one correlate between *in vitro* investigations and behavior *in vivo*.[89,137] The effect of hIAPP depends on the lipid to peptide ratio, the lipid composition, ionic strength and the pH. Many commonly used model systems that lack cholesterol contain a much higher percentage of anionic lipids than is found in the β-cell membrane.[138] These are important factors since cholesterol modulates hIAPP/membrane interactions and high percentages of anionic lipids significantly promote IAPP/membrane interactions.[139]

Toxic hIAPP aggregates cause pancreatic islet inflammation. hIAPP-induced β-cell proteotoxicity is linked to local islet inflammatory processes.[7,122,140,141] Activation of the inflammasome by hIAPP aggregates can contribute to local islet inflammation and β-cell dysfunction.[7,131] Inflammasomes are multiprotein intracellular assemblies that sense a diverse range of pathogenic stimuli and regulate the production of active caspase-1. In turn, caspase-1 activates the pro-inflammatory cytokines interleukin-1b (IL-1b) and IL-18 by cleaving of their pro-forms into mature sequences. The role of IL-1b in hIAPP-induced β-cell death and dysfunction is currently a major focus in the field.[7,131] The depletion of islet-resident macrophages, key modulators of inflammation, has been shown to improve glucose intolerance and increase islet amyloid accumulation, consistent with reports that amyloid fibrils are not toxic; suggesting that toxicity may be due to the activation of phagocytic cells that attempt to remove cytotoxic IAPP aggregates from the islet.[141] Interdisciplinary approaches combining studies in human diabetic pancreas, mouse models, cell physiology and molecular biophysics show that toxic prefibrillar form(s) of hIAPP (and not nontoxic monomers and not amyloid fibrils) are ligands of RAGE, and that hIAPP-induced upregulation and activation of RAGE leads to the induction of NADPH (nicotinamide adenine dinucleotide phosphate) oxidase (NOX), production of reactive oxygen species (ROS) and subsequent oxidative stress, a proximate mechanism preceding the development of β-cell inflammation, dysfunction, and ultimately, cleavage of caspases, and β-cell apoptosis.[2,110] A direct relationship between loss of β-cell area and β-cell stress, apoptosis and RAGE expression; and a lack of correlation between these parameters and islet amyloid area in a Tg hIAPP mouse model, support *in vitro* and *in vivo* findings that toxic pre-amyloid hIAPP intermediates are more deleterious than amyloid fibrils.[2] Macrophage chemo-attractants (Ccl2, Cxcl1 and Cxcl2) and other pro-inflammatory mRNA transcripts (Il1b and Il18) in the pancreas of Tg hIAPP mice with islet amyloidosis were significantly upregulated, while inhibition of hIAPP/RAGE interactions protected pancreatic islets, cells and Tg hIAPP mice from IAPP-induced stress, inflammatory gene

expression, metabolic dysfunction, islet amyloid deposition and loss of β-cell area.[2] Together, these findings establish RAGE as a mediator of pathological β-cell signaling during islet amyloidosis in T2D, and highlight the interaction of RAGE and pre-amyloid hIAPP intermediates as a primary target for β-cell preservation in metabolic disease.

Insulin Misfolding and Other Signals of Proteotoxicity in Diabetes

IAPP is co-produced and co-secreted with insulin by pancreatic β-cells. The biosynthesis of insulin by β-cells, like that of IAPP, is a multistep process, and mutations in the pre-proinsulin gene, or disruptions in posttranslational modifications at any major stage, can result in the formation of proteotoxic aggregates and activation of cellular stress mechanisms leading to β-cell toxicity and diabetes.[142–146] Insulin is initially translated in the cytosol as a pre-prohormone (pre-proinsulin), which is translocated across the ER membrane where it is enzymatically cleaved to form proinsulin.[147] Misprocessing during this early step results in the accumulation of pre-proinsulin in the juxtanuclear compartment and induction of heat shock protein 70 (HSP70), which promotes human β-cell apoptosis.[147] Inside the oxidizing environment of the ER, generation of three evolutionarily conserved disulfide bonds facilitate the proper protein folding of proinsulin (PI). Disruption of this key process leads to PI misfolding and its aggregation into cytotoxic species. For example, in the male *Akita* murine model of diabetes, a PI-C(A7)Y mutation in the A chain of PI prevents the formation of a critical disulfide bond, leading to proteotoxic aggregation, ER stress, increased XBP1 mRNA splicing, β-cell dysfunction, and the development of postnatal diabetes within weeks.[143,148] Proper posttranslational modification of the A-chain epitope of PI is also required for β-cell recognition and induction of adaptive immune attack by human T-cells.[149,150] Taken together, these data highlight the importance of posttranslational modification in insulin maturation and signaling, and indicate that defects in insulin processing can lead to PI proteotoxicity and/or autoimmunity, and subsequent β-cell apoptosis.

Elevated levels of PI have been shown to be correlated to the degree of β-cell secretory impairment in T2D patients.[151] In the β-cell, transport-competent secretory peptide precursors, including PI, are regulated by autophagy, whereas efficient clearance of transport-incompetent mutated forms of PI by alternative degradative pathways may be necessary to avoid β-cell proteotoxicity.[146,152,153] Insulin resistant *Akita* and *db*/*db* mice, as well as Zucker diabetic fatty rats display increased autophagosome flux, and disrupting autophagy in these models drives β-cell UPR stress and the progression of diabetes.[154–157] Reduction of PI autophagic degradation increases its residency in the secretory pathway and enhances its secretion in response to stimuli.[152,154,158] The production and overproduction of insulin in and of itself, drives cellular stress. Specifically, mitochondrial ROS have been implicated as an obligatory signal for GSIS,[159–162] and pharmacological inhibition of ROS diminishes insulin secretion. In the context of glucolipotoxicity, in which basal insulin hypersecretion and pronounced impairments in GSIS are known to occur, ROS stimulates insulin secretion from β-cells in a concentration-dependent manner.[163] This phenomenon is bidirectional, as NOX, a major driver of ROS production, is upregulated prior to β-cell dysfunction.[163] These reactive molecules, which play an adaptive role in cellular oxidative signaling, must be rapidly removed or else they elicit β-cell oxidative stress and metabolic defects.[163] Insulin stimulated insulin secretion has also been demonstrated in pancreatic β-cells,[164,165] providing another feed-forward mechanism by which augmentation of insulin production by β-cells may promote increased oxidative stress, further insulin secretion, more pronounced proteotoxicity and ROS production in a deleterious cycle, ultimately promoting β-cell failure and diabetes.

Conclusion

Protein aggregation and amyloid formation have long been recognized as key events in a range of neurodegenerative diseases, including AD and Parkinson's disease, and in the systemic amyloidoises, but their role in T2D is less appreciated. The various investigations highlighted in this review develop a strong case for the role of aberrant protein misprocessing, modification and aggregation in the pathophysiology of T2D. Aggregation of IAPP to form islet amyloid contributes to β-cell death and dysfunction in T2D, while aggregation of misprocessed insulin has been implicated to contribute to proteotoxicity in the disease. The co-secretion of insulin and IAPP suggests the potential forging of shared molecular and biochemical mechanisms of toxicity in pathological microenvironments. AGE formation is likely to play a significant role in proteotoxicity in T2D, given that the diabetic milieu is favorable for the generation of AGEs. AGE formation is thought to play a role in other protein deposition diseases and engagement of RAGE with toxic aggregates derived from different amyloidogenic proteins may provide a common unifying theme. It is not known whether RAGE plays a role in β-cell function; more work is needed to determine whether acute activation of RAGE in β-cells may be beneficial. But chronic activation of RAGE by its ligands, including those linked to protein aggregation or AGEs, can be detrimental. Compounds that interfere with the interaction of RAGE with its ligands, or suppress RAGE-mediated

cellular signaling triggered by binding of RAGE ligands, especially in the setting of pathological ligand conformation and/or concentration, may thus have therapeutic potential for a range of inflammatory diseases, including diabetes.[2,166]

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