Islet amylold in type 2 diabetes:

The role of chaperones in endoplasmic reticulum stress and amyloid formation in pancreatic β-cell

Lisa Cadavez Trigo

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ISLET AMYLOID IN TYPE 2 DIABETES:

The role of chaperones in endoplasmic reticulum stress and amyloid formation in pancreatic β-cell

Presented by:

Lisa Cadavez Trigo

Candidate for the degree of Doctor in Biomedicine

Under the supervision of:

Dr. Anna Novials Sardà

Dr. Joel Montanè Mogas

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A mi família,

António, Maria Luísa y

Raquel

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FIGURES

TABLES

ATF3	Activating transcription factor 3
ATF6	Activating transcription factor 6
BiP	Heavy chain binding protein
BSA	Bovine serum albumin
Ca^{2+}	Calcium
cDNA	Complementary deoxyribonucleic acid
CHOP	CCAAT/enhancer-binding protein (C/EBP) homologous protein
CMV	Cytomegalovirus
CPE	Carboxypeptidase E
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DTT	Dithiothreitol
ECL	Enhanced chemiluminesce
eIF2α	Eukaryotic translation initiation factor 2α
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ERAD	Endoplasmic reticulum associated degradation
FBS	Fetal bovine serum
FFA	Free fatty acid
GLUT4	Glucose transporter four
GRP78	Glucose-regulated protein 78
GSIS	Glucose-stimulated insulin secretion
HFD	High fat diet
HGP	Hepatic glucose production
hIAPP	human Islet Amyloid Polypeptide
Hsp	Heat shock protein
IAPP	Islet amyloid polypeptide
IFG	Impaired fasting glucose
IGF	Insulin-like growth factor
IGT	Impaired glucose tolerance

INS1E	Rat insulinoma cell line
IRE1	Inositol requiring enzyme 1
JNK	Jun N-terminal kinase
K_{ATP}	ATP-sensitive K+ channels
KRBH	Krebs-Ringer bicarbonate buffer
MIN6	Mouse insulinoma cell line
mM	Millimolar
MODY	Maturity onset diabetes of young
MOI	Multiplicity of infection
mRNA	Messenger ribonucleic acid
mTOR	Mammalian target of rapamycin
NGT	Normal glucose tolerance
Ob	Obese
P/S	Penicillin/streptomycin
PA	Palmitate
PBA	4-phenyl butyric acid
PBS	Phosphate buffered saline
PC1	Proinsulin convertase 1
PC2	Proinsulin convertase 2
PDI	Protein disulphide isomerase
PERK	Pancreatic eukaryotic initiation factor 2α kinase
PVDF	Polyvinylidene fluoride
qRT-PCR	Quantitative real-time polymerase chain reaction
rIAPP	Rat Islet Amyloid Polypeptide
RNA	Ribonucleic acid
rpm	Revolutions per minute
RPMI	Roswell park Memorial Institute medium
RT	Reverse transcriptase
S1P	Site 1 protease
S2P	Site 2 protease
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Sec61	Secretory 61 protein

SEM	Standard error mean
SERCA	Sarcoplasmic/endoplasmic reticulum Ca^{2+}
siRNA	Short interfering RNA
SRP	Signal recognition particle
T1D	Type 1 Diabetes
T2D	Type 2 Diabetes
TAE	Tris-acetate-EDTA
TbsT	Tris buffered saline-tween20
TG	Triglyceride
ThioS	Thioflavin S
Thp	Thapsigargin
Tris	Tris (hidroximetil) aminometano
TUDCA	Taurine-conjugated ursodeoxycholic acid
UPR	Unfolded protein response
WT	Wild-type
XBP1	X-box binding protein 1
β-cell	Beta cell

Chapter I.
INTRODUCTION

1. DIABETES MELLITUS: OVERVIEW OF THE PATHOGENESIS

Diabetes is a chronic metabolic disease characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both. The chronic hyperglycemia of diabetes is associated with long-term damage, dysfunction, and failure of different organs, especially the eyes, kidneys, nerves, heart, and blood vessels. There are four main types of diabetes, namely Type 1 Diabetes (T1D), Type 2 Diabetes (T2D), Gestational Diabetes (GD) and, Maturity-onset diabetes of young (MODY).

Type 1 Diabetes Mellitus

T1D which appears predominantly in childhood or adolescence, corresponds to ~10% of the cases of diabetes mellitus, in which patients produce little or no insulin due to an autoimmune destruction of their own insulin-producing β-cells. T1D is also referred as insulin-dependent diabetes mellitus and is mortal unless there is insulin treatment [1].

Gestational Diabetes Mellitus

GD is a condition characterized by high blood glucose levels that was not present before pregnancy and is present in ~4% of all pregnancies. During this period, the insulin levels need to be much higher than normal however, the pancreas is not able to make enough insulin to drop glucose on plasma. Several factors are associated with the appearance of GD, being the most common the overweight or obesity and family history of GD and T2D. In addition, patients that developed GD are considered more vulnerable of developing T2D [2].

Maturity-onset diabetes of young

Recent studies suggest a genetic component in the development of diabetes. Monogenic forms (as opposed to T1D and T2D that are polygenic) have been identified, being the most common commun the MODY, which involves mutations in several different genes, being the most frequent forms the MODY1, MODY2 and MODY3 due to mutations in the glucokinase (*GCK)*, hepatocyte nuclear factor-1α *(HNF1α)* and hepatocyte nuclear factor-4α (*HNF4α*) genes, respectively. MODY is typically diagnosed in children, adolescents or early adulthood and

corresponds to a primary defect in insulin secretion, associated with dysfunction in the pancreatic β-cell [3].

Type 2 Diabetes Mellitus

T2D is considered the most common form and responsible for 90% of the overall diabetes prevalence. Generally it appears later on in life and is formerly called non-insulin dependent diabetes mellitus. It has been associated with a reduction in β-cell mass [4] and peripheral resistance to the action of insulin. Previously, T2D was only diagnosed in older individuals; however, more children and adolescents are being diagnosed with diabetes. This shift in the onset of T2D correlates with an increasing population of overweight children.

Nowadays, the incidence of T1D is predicted to remain steady; however the number of T2D cases is expected to dramatically increase. Indeed, T2D has become a very common and fast growing epidemic disease with a social and economic impact. Today, around 250 million people worldwide are living with diabetes and by 2030 this total is expected to increase to over 400 million [5]. This augment will be more evident in developing countries; however the regions with greatest potential increases are the low- and middle-income countries, where diabetes rates could rise to 2 or 3 times more [6] (**Figure 1**). Over the past decades, not only diet quality and quantity have experience profound modifications, but also a reduction in physical activity. For that, the whole population have conducted to an increase in the frequency of diabetes and its complications with a significant impact for health systems [7].

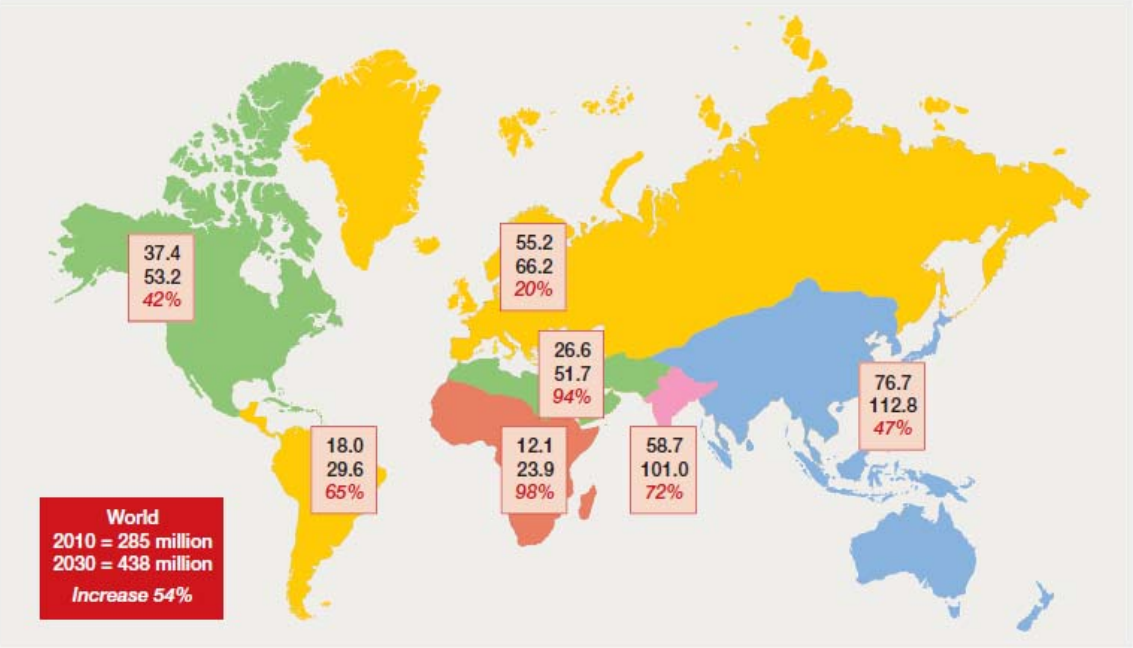


Figure 1. Global projections for the diabetes epidemic: 2010-2030 (millions) (From Medicographia, 2011).

This increase points to a serious problem for society caused by a drop in work productivity, early retirement and premature death. Although diabetes has become the fourth or fifth leading cause of death, many governments still remain unaware of the real magnitude and the risks for the rapidly rising in diabetes in their own countries. Thus, diabetes is certain to be one of the most challenging health problems in the 21st century [6].

The demanding health problem is often accompanied by a comprehensive list of chronic complications shared by both T2D and T1D, affecting almost all tissues which including kidney failure (nephropathy), heart diseases stroke and hypertension, blindness (retinopathy) and nervous system disease (neuropathy) and amputation. Nevertheless, unlike T1D which is a chronic autoimmune disorder that selectively destroys the pancreatic β-cells within the islets of Langerhans leading to a lack of insulin, T2D is classified as an “insulin-independent” disorder and the pathogenesis of this disease is associated by both insulin resistance and β-cell dysfunction that has impaired production of insulin.

1.1. THE ENDOCRINE PANCREAS

1.1.1. The role of pancreas

The human pancreas is a complex organ that lies in the upper abdomen behind the stomach making part of the digestive system. As a glandular tissue, the pancreas is organized in two compartments each one with different functions:

The exocrine compartment is responsible for the process of digestion by the acinar and ductal cells, which produces digestive enzymes and secretions and drain into the duodenum via de pancreatic duct. The exocrine compartment comprises more than 95% of the pancreatic mass.

The endocrine compartment is involved in maintaining blood glucose levels, through the production of hormones by the pancreatic islets, which are the main structures of the endocrine compartment and comprise 5% of the total pancreatic cell mass.

1.1.2. Morphology of the pancreas

The anatomy of the pancreas consists basically in four regions: head, neck, body and tail. The head is the broadest part of the gland and is in the curvature of the duodenum; the neck, which lies between the head and the body; the body with a canonical shape is localize behind the liver and the small intestine and finally the tail, is the narrowest part and is at the left, in contact with the posterior surface of the stomach and spleen (**Figure 2A**).

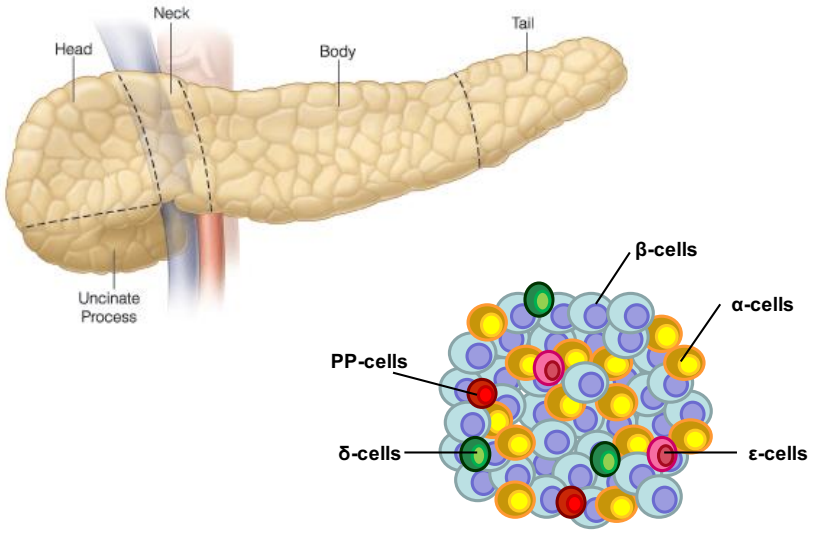


Figure 2. Structure of the pancreas. (A) General morphology of the pancreas. **(B)** Schematic image of the structure of human pancreatic islets. (*From ACS Surgery: Principles and practice online*).

The endocrine compartment is composed of millions of pancreatic islets, which are distributed throughout the exocrine tissue as small clusters (**Figure 2B**). They are localized along with the ducts of the pancreas, with a higher density in the tail when compared with the head and body.

1.1.3. The structure of the Islets of Langerhans

The islets of Langerhans were discovered in 1869 by Paul Langerhans and described as small islands rich in blood cells. There are around 1-2 million islets in humans and all islets consist of five different types of cells: the β-cells, which are the most abundant, constitute more than 70% of the islet cells mass and produce insulin and amylin; the α-cells, which make up 20% of the total and secret glucagon; the δ-cells produce somatostatin, and make up 3-10% of the total islet cells; the PP-cells produce the pancreatic polypeptide, and make up 3-5% of the total islet cells, whereas the ε-cells produce the hormone grelin and account for about 1% of islets cells (**Figure 2B**).

In the middle of the last decade, diabetes researchers have discovered that the internal structure of the human pancreatic cells is significantly different than the islets in rodents, being the

β-cells the most abundant cell type in the islets of all species [8-9]. In humans, all five cell types are spread through the islet with no particular order of distribution, resulting in the β-cells having direct contact with the other cells. However, in rodents, β-cells are located in the middle of the islet and surrounded by a layer of the rest of cell types (**Figure 3**) [9-10]. Although, β-cells are the most abundant cell type in islets of all species, the proportion of β-cells is higher in mouse islets when compared to human cells. Moreover, this particular architecture observed in human islets may affect the way that all type of cells interact to each other in order to respond to glucose and their ability to survive [10].

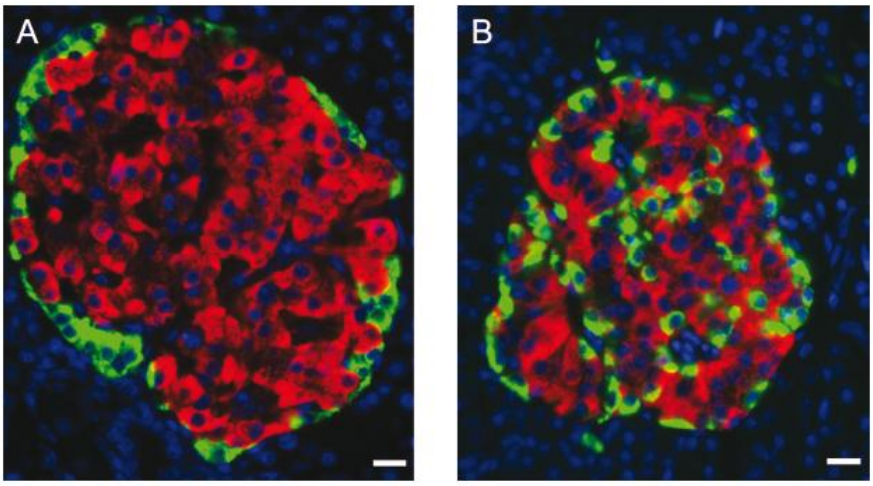


Figure 3. Immunofluorescent labeling of rat and human islets. Pancreatic sections from adult rat **(A)** and human **(B).** Note that in rat islets, α-cells surround β-cells, which is not the case in human islets. α-cell (green), β-cell (red), nuclei (blue) [9].

1.2. BIOSYNTHESIS AND SECRETION OF INSULIN IN PANCREATIC β-CELLS

1.2.1. Insulin biosynthesis

Insulin, which is produced by the pancreatic β-cells, is the most potent anabolic hormone with powerful effects on a vast range of physiological process involving mitogenic and metabolic events. One of the most important function of insulin is based on the regulation of glucose

homeostasis, requiring for that, a tightly coordination between insulin secretion and action [11]. Insulin is stored in granules and secreted in response to increasing plasma glucose levels.

Insulin is a hormone of 51 aminoacids synthesized as a precursor protein, called preproinsulin, which is formed by 110 aminoacids and a signal sequence, in the endoplasmic reticulum (ER) membrane. The co-translational polypeptide is then translocated into the ER lumen and converted to proinsulin by removal of the signal sequence. Upon glucose stimulation, proinsulin is then folded and disulfide bonds are formed by an enzyme called protein disulfide isomerase (PDI) and the connecting peptide (C-peptide) is cleaved by the membrane-associated endoproteases PC1/3 and PC2 as well as carboxypeptidase E [12].

1.2.2. Mechanisms of insulin release in β-cells

Glucose-stimulated insulin secretion (GSIS) is the principal mechanism of insulin release. When blood glucose rises, glucose is transported into the β-cell by facilitated diffusion through glucose transporter-2 (GLUT2) [11]. After a short period of time, glucose is metabolized leading to an increase production of ATP in the cytosol that in turn deactivates ATP sensitive K^+ channels (K_{ATP} channels). The closure of K_{ATP} channels is the main responsible for the depolarization of the plasma membrane, causing an influx of Ca^{2+} through the opening of the voltage-gated Ca^{2+} channels. The increased Ca^{2+} levels in the cytoplasm trigger the release of insulin (**Figure 4**). This process of insulin secretion is called the “first phase” or triggering pathway, and is followed by a “second phase” or amplification pathway, which occurs post K_{ATP} channels and where is not necessary a large increase of [Ca^{2+}] for insulin exocytosis, but it is sufficient to accelerate the granules mobilization [13]. Accordingly, the first phase of insulin secretion is thought to be maximal 4 minutes after glucose stimulation and the second phase is characterized by an increasing rate of secretion that plateaus at approximately 30 minutes [14]. Thus, both phases of insulin secretion are synergistic and therefore not strictly independent.

In addition to glucose, other substances are known to regulate insulin secretion. Is the case of aminoacids arginine and leucine, free fatty acids, acetylcholine, glucose-dependent insulinotropic polypeptide (GIP), glucagon-like peptide-1 (GLP-1), and several other agonists [15].

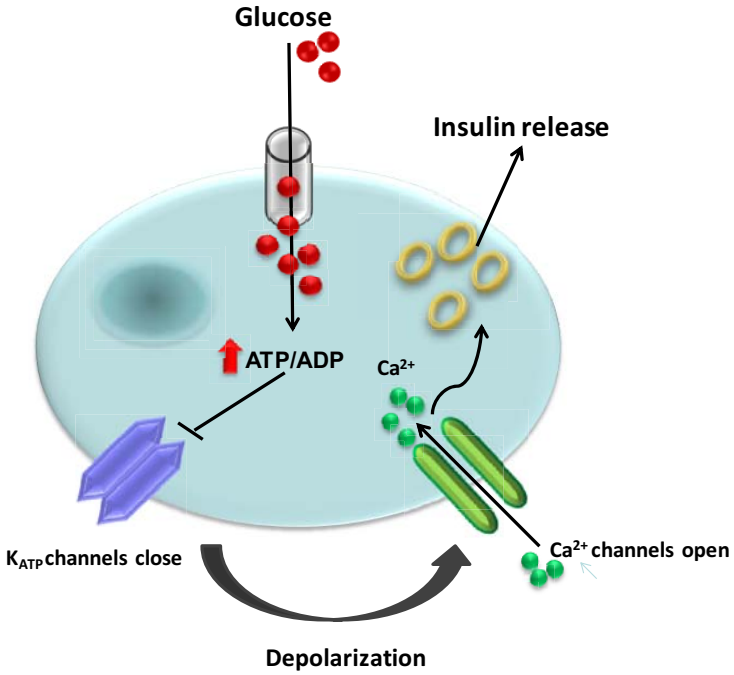


Figure 4. Glucose-stimulated insulin in pancreatic β-cells.

1.3. PRINCIPLES OF THE PATHOGENESIS OF TYPE 2 DIABETES

The genetic and molecular basis of the pathogenesis of T2D is not completely elucidated; however a growing body of evidence has reported that the progression from normal to impair blood glucose levels regulation in individuals with T2D, is mostly influenced by insulin resistance and β-cell dysfunction leading to a state of hyperglycemia [16]. Over the past years, a large number of considerations have been given to these two events:

1) Some groups have suggested insulin resistance as the first abnormality to emerge followed by β-cell dysfunction, which is characterized as the late event that appears as a consequence of the prolonged and increased secretory demand of insulin [17].

2) Others studies have proposed the drop in β-cell function as the main requisite to the transition of normal glucose tolerance to hyperglycemia [18-19]. Furthermore, insulin resistance alone (in the non-β-cell target tissues of the hormone) cannot cause diabetes without β-cell failure [20].

This disagreement is due mostly to an isolated evaluation, not considering that glucose homeostasis is significantly dependent on a synergistically system that includes each state. Moreover, the preservation of normal glucose homeostasis depends on a precisely interaction between insulin sensitivity of peripheral tissues (muscle, adipose and liver) and insulin secretion. Therefore, the development of T2D requires a defect in insulin action and insulin secretion. Both features will be discussed afterwards.

1.3.1. Insulin resistance

Insulin resistance is defined as the condition where insulin-responsive tissues fail to respond properly to normal levels of circulating insulin [21]. Insulin resistance has been shown to be the best predictor of the disease and also the central and underlying feature of T2D. It is known that insulin resistant individuals have normal or slightly elevated fasting plasma glucose in the presence of elevated insulin concentrations, and they often exhibit prolonged postprandial hyperglycemia (**Figure 5**).

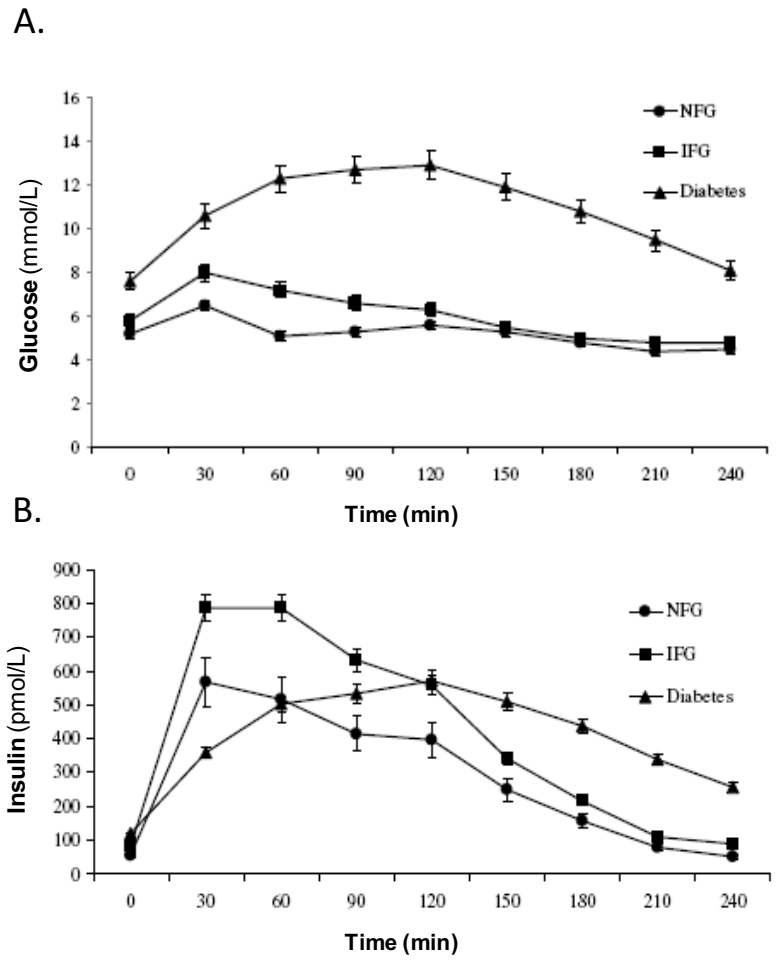


Figure 5. Glucose and insulin responses during a standard liquid meal. Plasma glucose **(A)** and plasma insulin **(B)** values by timepoint during a standard liquid meal tolerance test according to fasting glucose tolerance status. NFG = normal fasting glucose; IFG = impaired fasting glucose [22].

Insulin resistance affects the action of insulin in several target tissues, but muscle, liver and adipose tissue seem to play the major roles in insulin-induced glucose clearance. In effect, there is a reduction on insulin glucose uptake by skeletal muscle and adipose tissue and an impaired suppression of hepatic glucose production (HGP). The contribution of these tissues to the evolution of T2D is described below (**Figure 6**):

Adipose Tissue:

The most commonly associated disorder linked with the onset of insulin resistance is obesity. As the fat mass of obese individual's increases, the concentration of inflammatory

mediators, termed adipokines produced by the adipocytes rises influencing insulin secretion and insulin desensitization [23]. In addition, evidences have demonstrated that an increased mass of fat cells are able to release high quantities of free fatty acids (FFA) into the circulation. In the case of obese subjects, fat cells are already overloaded with FFA in the form of triacylglycerols (TG). As a result, the uptake of excess TG-FFA is diminished, leading to its accumulation in peripheral tissues such as skeletal muscle, liver and pancreatic β-cells causing an impairment in insulin sensitivity in those tissues (in the case of β-cells, to an impairment in insulin secretion in response to glucose) [24-25].

Muscle:

Skeletal muscle is considered the central organ of glucose uptake in the postprandial state and the main responsible for the development of insulin resistance in T2D, being the impaired glycogen synthesis one of the earliest metabolic defects [26]. However, other abnormalities in insulin action on skeletal muscle metabolism are consistently observed in patients with T2D. About 80-90% of patients with T2D are obese, and as mentioned above, obesity itself causes skeletal muscle insulin resistance via secretion of adipokines, inflammatory mediators, and growth factors. Another possible mechanism for the development of insulin resistance in muscle has to do with abnormal activation of the insulin-signal cascade, including decreased of insulin receptor 1 (IRS-1) protein content, impaired IRS-1 phosphorylation, reduced phosphoinositide 3-kinase (PI3K) activity, or altered protein expression of the regulatory subunit of PI3K. Additionally, alterations in the expression or the translocation to the plasma membrane of GLUT4 have been implicated as a potential pathogenic mechanism [27].

Liver:

Although the liver accounts for only ~30% of the whole-body glucose metabolism, hepatic insulin resistance plays an important role in the pathogenesis of T2D. After an overnight fasting, the liver of healthy subjects produces glucose in order to supply the needs of the brain, which is mainly dependent of the total body mass and the grade of peripheral insulin utilization. HGP is suppressed by insulin, which is secreted after meal ingestion. However, the liver of T2D subjects fails to recognize the presence of insulin, leading to an overproduction of glucose by the hepatocytes. Several lines of evidences have revealed new others factors that contribute to the increased rate of

HGP including elevated circulating glucagon levels, decreased activity of glucokinase enzyme and hepatic sensitivity to glucagon [28]. Besides, the elevated circulating free fatty acids are also reported to stimulate hepatic gluconeogenesis which is responsible for high glucose production in diabetic patients [24].

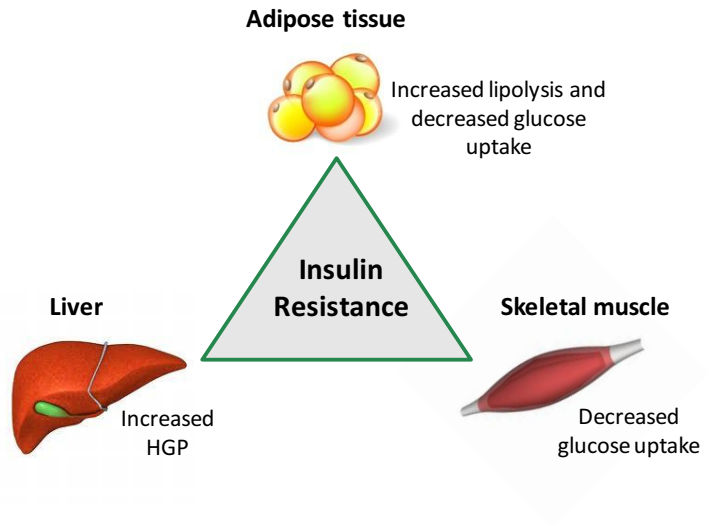


Figure 6. A schematic overview of the contribution of peripheral tissues for the progression of insulin resistance in the pathology of T2D.

In summary, insulin resistance affects the action of insulin in major target tissues such as muscle, adipose and liver. In fact, the normal β-cell reacts to the state of insulin resistance by increasing the secretion of insulin to the bloodstream, resulting in hyperinsulinemia. This physiological state is not only a compensatory response to insulin resistance, but also the leader cause of the defects in insulin action in several tissues, predominantly in the β-cells which, eventually become exhausted in the course of time and lose their ability to secrete insulin [29]. The progressive decline of β-cell function precedes the development of hyperglycemia after the diagnosis of T2D. Moreover, the contributions of other tissues for this condition, and not only those described above, are also important and cannot be disregarded (**Figure 7**).

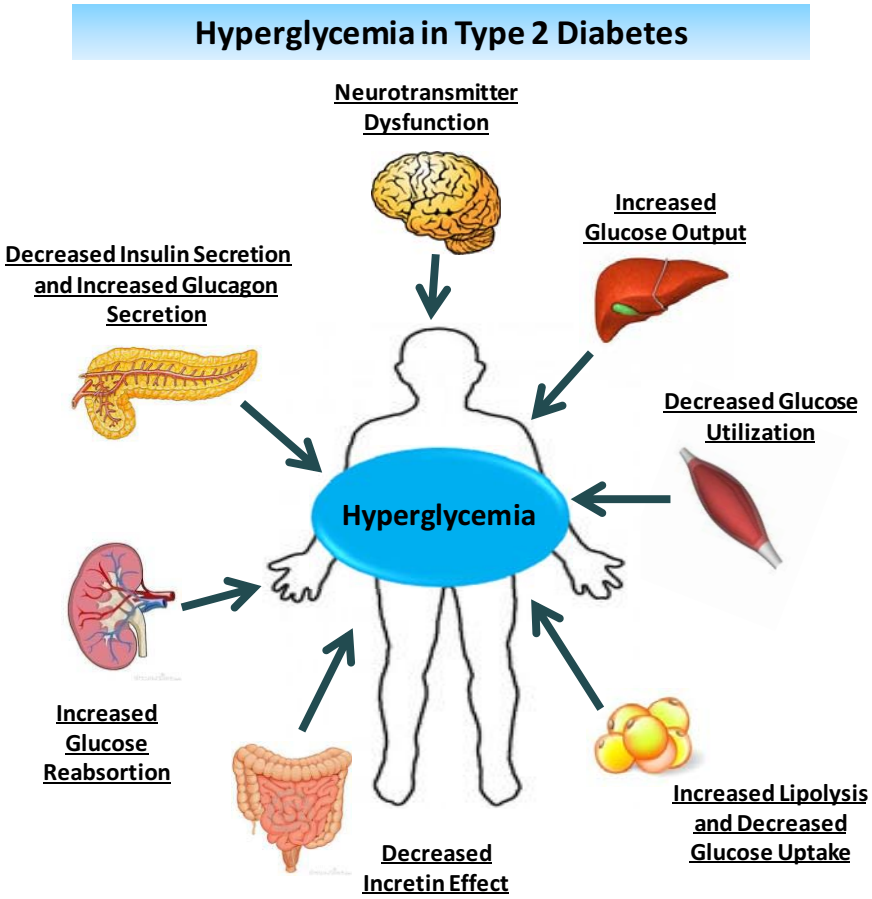


Figure 7. The contribution of several tissues for the appearance of hyperglycemia in T2D.

1.3.2. From normal β-cell function to β-cell dysfunction

The main role of β-cell is to synthesize and secrete insulin in order to maintain circulating glucose levels within physiological range. In T2D, β-cell dysfunction plays a determinant role, not only in the pathogenesis of the disease, but also in disease progression over time. Although, β-cell dysfunction is initiated years before clinical symptoms appear, and long before the onset of pre-diabetes, it is estimated that at diagnosis, β-cell function is already reduced by 50-60% (**Figure 8**). Several studies in T2D patients have shown that β-cell dysfunction occurs primarily with a defective in the first phase of insulin secretion [30]. In the second phase, insulin secretion is also affected latter during the progression of the disease, however it could be re-establish in some patients under the control of glucose concentration in the blood.

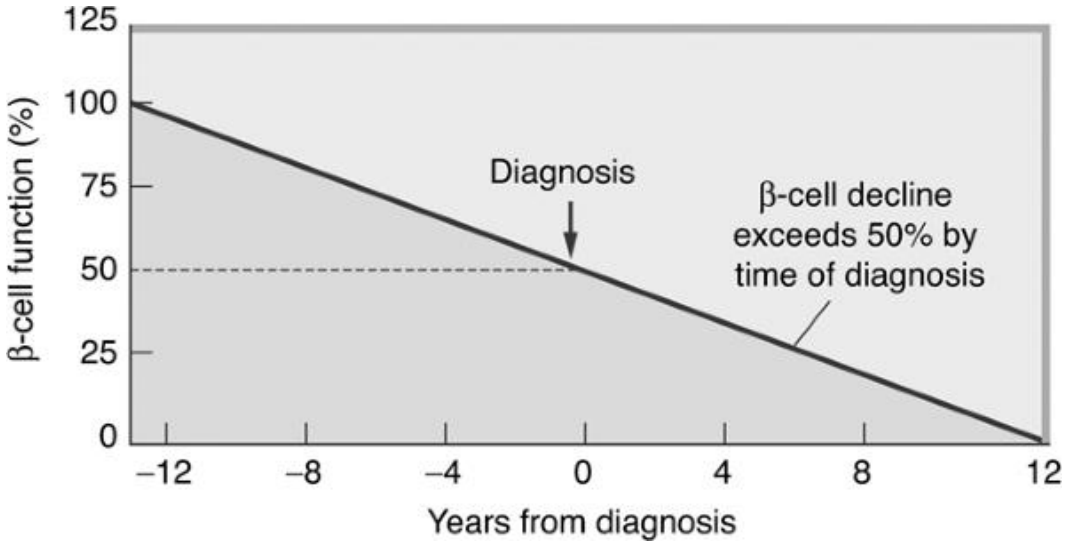


Figure 8. β-cell function decline over time. Patients with T2D, have already lost 50% of their β-cells upon diagnosis (from Diabetes Management in the Primary Care Setting, 1st Edition).

During the last decade, the reduction in β-cell function has been attributed mostly to a decrease in β-cell mass; however its precise role for the etiology of T2D remains controversial due to a lack in longitudinal studies. In spite of this, several studies in patients with T2D have shown a significant reduction ranges from ~20% in some studies to ~65% in others [31-32]. Although, the reasons underlying this β-cell deficit are not clearly understood, some factors responsible for this decline have been suggested; for example metabolic abnormalities (glucotoxicity and lipotoxicity) may induce apoptosis, hormonal changes (inadequate incretin secretion and action), aging and genetic anomalies [33]. Additionally, a growing body of evidences have shown the contribution of an inefficient insulin processing [34-35] and the hypersecretion of islet amyloid polypeptide (IAPP) together with amyloid deposition for the establishment of T2D [36-37]. The contribution of the majority of factors mentioned above will be further described in the next sections of the Introduction.

The loss of β-cell mass seems to be present in mostly of T2D patients obtained at autopsy, however the question whether loss of mass or loss of function underlies the β-cell defects in T2D is not likely to be conclusively. Theoretically, the insulin secretory defect could result from either defects of β-cell function or a reduction in β-cell mass. Some lines of evidences indicate that T2D associates with either no change or < 30% reduction in β-cell mass. Moreover, the secretion defect

is more severe than can be accounted for only by the reduction in β-cell mass. It therefore appears that insulin secretory defect in T2D does not primarily result from insufficient β-cell mass but rather from an impairment of insulin secretion that contributes to the progressive deterioration of glucose homeostasis. However the number of β-cell tend to be less eventually, as long as β-cells are able to secrete insulin as much as necessary to compensate insulin resistance, the glucose tolerance will remain normal.

2. TYPE 2 DIABETES AND ISLET AMYLOID POLYPEPTIDE (IAPP)

One of the major causes involved in the process of β-cell deterioration is the presence of amyloid deposits in the pancreas. The process of amyloid deposition is a remarkable physiopathological finding in individuals with T2D (**Figure 9**). The term amyloid emerged from the Latin word *amylum* which means starch. For a long time it was thought that these deposits were starch-like, but later on, it was discovered that they were actually a mass of proteins with a particular beta sheet structure. From that, pathologies with conformational changes in normally soluble proteins or peptides that result in the formation of intermolecular hydrogen bonds, beta sheet conformation and fibril formation are namely amyloidosis. Apart from T2D, these conditions have been also implicated in different human disorders including such debilitating diseases such as Alzheimer's disease, Parkinson's disease and Huntington's disease [36].

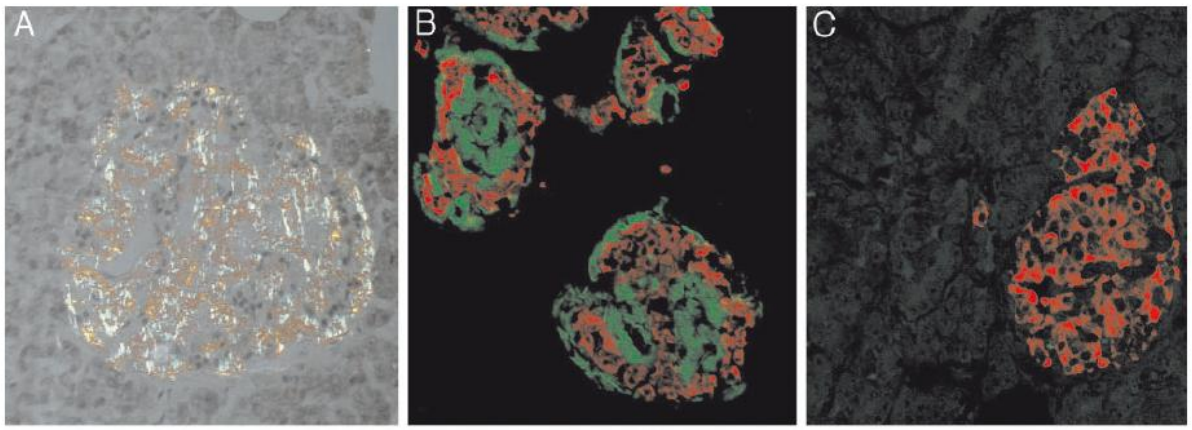


Figure 9. Islet amyloid deposition in islets from an individual with T2D. Islets stained with Congo Red and viewed under partially cross-polarized light. Islet amyloid is visible as areas of *pink/brown* staining and *apple green* birefringence **(A)**. A pancreatic islet from an individual with T2D showing islet amyloid deposition by thioflavin S staining (*green*) and residual β-cells with insulin immunostaining (*red*) **(B)**. No amyloid is present in a pancreatic islet from a nondiabetic individual; insulin immunostaining shows β-cells (*red*) **(C)** [38].

The contribution of IAPP in T2D is still controversial; several studies question whether amyloid deposition is a cause or a consequence of islet decline and whether it occurs intra- or extracellularly [39]. However, numerous evidences correlate the role for IAPP with the severity of the disease. The facts indicate that amyloid deposits are seen in ~90% of patients with T2D at autopsy and can possibly correspond to late stages of the pathology [40-41].

2.1. GENERAL FEATURES OF IAPP: SYNTHESIS, SECRETION AND FUNCTION

Islet amyloid polypeptide (IAPP) also known as amylin is a normal product of pancreatic β-cells. It is stored along with insulin in secretory granules and co-released in response to nutrient stimuli. This 37- amino acid peptide was identified in 1987, although it had been in 1989 that the gene was isolated and characterized [42]. Nishi et al., located it in chromosome 12, containing three exons and two introns, which transcribed an 89- amino acid precursor, termed preproIAPP peptide with an amino-terminal signal sequence. This signal peptide is then cleaved from the precursor protein to generate a 67- amino acid propeptide termed proIAPP [43]. This peptide undergoes further post translational modifications, which include the formation of disulfide bridges between cysteine residues at position 2 and 7 and also the amidation of the C-terminal tyrosine. Therefore

the normal processing of proIAPP in β-cells begins with the cleavage at its COOH terminus by the convertase PC1/3 to produce the intermediate proIAPP, which is followed by the cleavage in the secretory granules by PC2 [44]. The remaining COOH-terminal is removed by the carboxypeptidase E (CPE) resulting in the mature 37- amino acid IAPP (**Figure 10**) [37, 44-45]. These prohormone convertases are as well responsible for proteolytic conversion of proinsulin to insulin, supporting the idea that the processing of proIAPP might also be impaired in T2D [46].

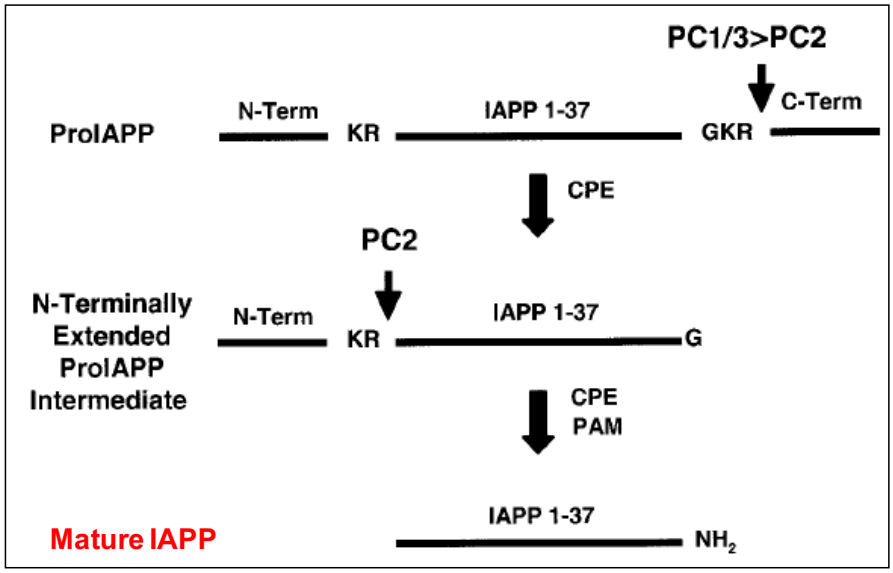


Figure 10. Proposed model for normal processing of pro-IAPP in islet β-cells. (a) Pro IAPP processing is initiated by cleavage at its COOH-terminus by either PC1/3 or PC2, although cleavage by PC1/3 is favored at this site. Cleavage of the NH2-terminally unprocessed proIAPP intermediate by PC2 then results in the production of IAPP (~4 kDa). After cleavage by PC1/3 or PC2, the remaining dibasic residues are likely removed by CPE and mature IAPP is formed by removal of Gly at the COOH-terminus and amidation at this site by the peptidyl amidating mono-oxygenase complex (PAM). (Adapted from Marzban et al, 2004) [45].

The IAPP primary structure is highly conserved between human, non-human primates and cats [47]. Its sequence appears to show a close homology among species in the carboxy terminal residues, however the residues 20 to 29 seems to exhibit some variation (**Figure 11**). IAPP has been shown to be secreted following stimulation with glucose and other secretagogues and in a high frequency pulsatile manner, similar to insulin [48]. In healthy humans, plasma levels of endogenous circulating IAPP are lower in the fasted stated (~4-8 pmol/l) and rise to 15-25 pmol/l after a meal,

correlating with levels of insulin [49]. However, unlike insulin, IAPP is not eliminated significantly in the liver but mostly through renal metabolism [50] and the plasma clearance rate has been shown to be slower than insulin.

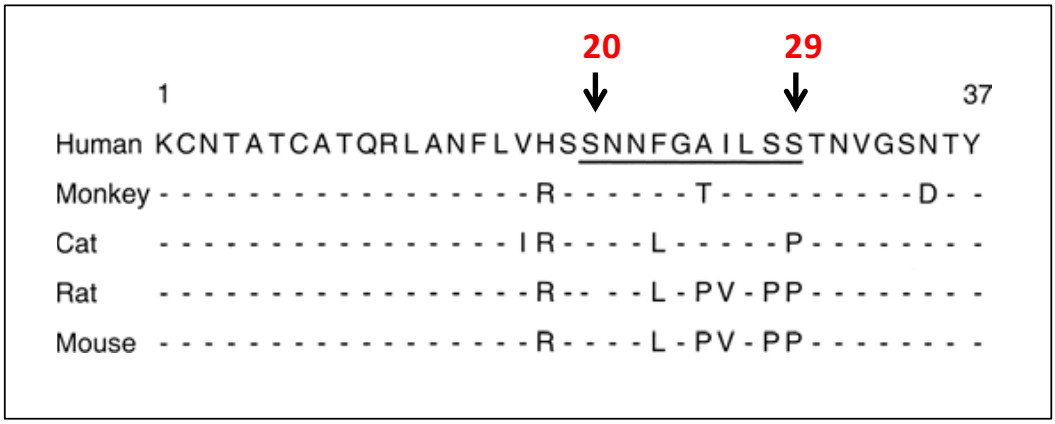


Figure 11. Species differences in the amino acid sequence of islet amyloid polypeptide. Amino acids in monkey, cat, rat, and mouse IAPP that are identical to the human sequence are denoted (-). The underlined sequence (amino acids 20-29) is thought to be critical for the formation of amyloid fibrils in humans (Adapted from Diabetes Mellitus: A Fundamental and Clinical Text 3rd Edition)

The function of IAPP has been suggested to be involved with glucose homeostasis. In general, this hormone inhibits gastric emptying and is important in controlling and delaying the rate of meal derived glucose. It has also been shown to inhibit glucagon secretion as well as somatostatin. Indeed, physiological concentrations of IAPP are responsible for the regulation in food intake and body weight. It has been shown *in vivo*, that administration of IAPP in rat brain is related with a reduction in food intake when compared with an IAPP antagonist [51]. Several others effects have been related including the regulation of renal filtration [52], calcium homeostasis [53] and vasodilatation [54]. Nevertheless, a critical role positions IAPP as the main responsible for the pathogenesis of T2D through formation of amyloid deposits and destruction of pancreatic β-cell.

2.2. MOLECULAR MECHANISMS OF AMYLOID FORMATION AND AGGREGATION

Although there has been considerable progress, the exact mechanism of abnormal aggregation of IAPP is still largely unknown; however several studies pointed the overproduction or mutations in IAPP as the main causes for amyloid formation [55]. The role of overproduction of IAPP is associated with the increased secretory demand for insulin due to insulin resistance and increasing hyperglycemia. Because IAPP and insulin are co-secreted in β-cell secretory granules, this increased production and secretion could result in accumulation and aggregation of IAPP. Many studies have reported that transgenic mice with overexpression of human IAPP develop islet amyloid deposits [56-57]. However, other studies not only contradicted this hypothesis but claim that overproduction of IAPP, even at levels several times higher than normally, per se, are not sufficient for islet amyloid formation [58]. To confirm these findings, Kahn et al demonstrated that non-diabetic obese and/or insulin resistant individuals with elevated IAPP production do not developed amyloid deposits. However, in both cases, only in the presence of some factors such as, genetic predisposition, high fat diet and obesity, IAPP might be critical for the development of extensive islet amyloid [56-57].

Another possible mechanism for amyloid formation concerns the mutations in the IAPP gene, by producing forms more fibrillogenic than the normal peptide. Based on several studies, the S20G mutation in the IAPP gene appears to be associated with an early onset and more severe form of T2D, due to a higher frequency in patients with T2D than in control subjects [59]. This discovery supports the idea that IAPP may play a role in the pathogenesis of T2D. In addition, there are at least two other mutations associated with T2D, which have been identified in the promoter region of the IAPP gene [60-61]. Still, others studies have failed to demonstrate any correlation between abnormalities in IAPP gene and the disease [62-63]. Although it is not possible to explain how the mutations interfere with the progression of amyloid formation, but it is possible that mutations may be contributors in some patients with T2D.

In addition, the existence of hydrophobic amino acids in the mid-portion of IAPP could also be responsible for its propensity to aggregate into β-pleated sheets. The residues at position 20-29 of the polypeptide chain have been determined to be the amyloidogenic region of the peptide. However, the proline substitutions in the 24-29 regions of rodent IAPP are thought to prevent

amyloid fibril formation completely [38]. Proline is a known to be β-sheet breaker and a total inhibition of amyloid formation was seen when substitutions of proline in the human wild-type 20-29 region were performed [64]. Additionally, several hypothesis indicate that the impaired processing of proIAPP may result in an elevated secretion of the NH_2-terminal IAPP precursor with a strong affinity for heparan sulfate proteoglycans which could eventually result in a generation of a nucleus for amyloid formation [55, 65].

2.3. IAPP TOXICITY

Aggregated IAPP has cytotoxic properties and is believed to be of critical importance for the progression in patients with T2D. Early studies by Howard et al. (1986) and by Clark et al. (1988) have shown that the formation of islet amyloid is strongly associated with reduction of insulin secretion and with loss of approximately 50% of the β-cell mass [66-67]. IAPP aggregation has been suggested to occur in a stepwise manner, with soluble monomeric IAPP forming oligomeric structures, protofibrils and eventually amyloid fibrils (**Figure 12**).

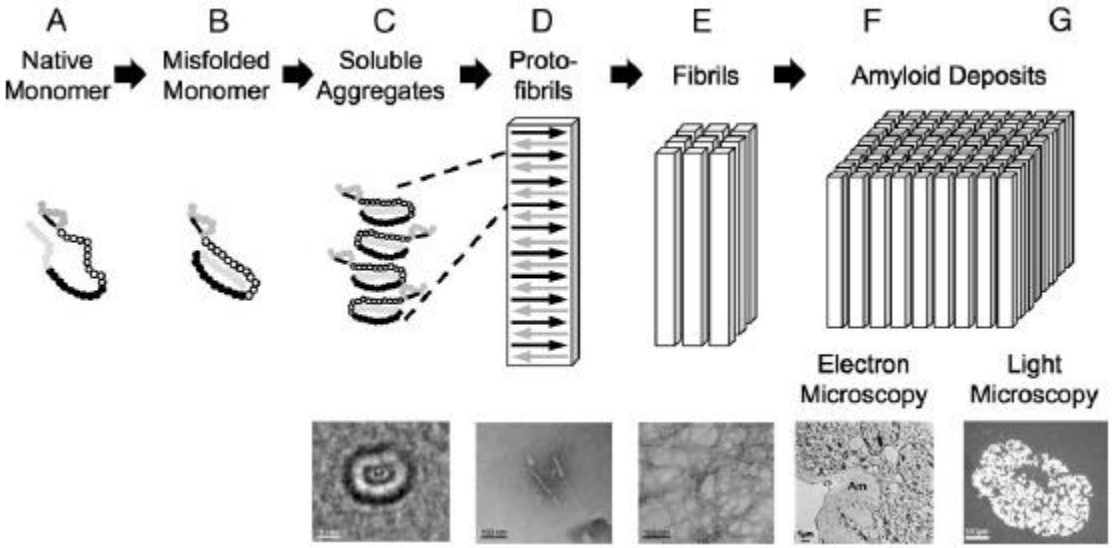


Figure 12. Model for islet amyloid fibril formation and cytotoxicity [38].

Initially there was general acceptance about the concept that the fibrillar form of IAPP are the toxic species [68]. Furthermore, studies have shown a strong correlation between islet amyloidosis and IAPP cytotoxicity and eventually β-cell death. Yankner laboratory (1994) have demonstrated that toxicity is mediated by IAPP fibrils by direct contact of fibrils with the cell surface causing DNA fragmentation, chromatin condensation and protuberances in the plasma membrane leading to islet cell apoptosis [69]. The common feature for IAPP fibrils lies on the classical cross β-structure, polymorphic and typically unbranched [70]. Additionally, *in vitro* studies have provided evidences that synthetic IAPP readily forms amyloid fibrils which allowed studying the overall morphology and formation [71-72].

Recently, literature has emerged suggesting that amyloid fibrils are less toxic than small oligomers formed by aggregates of IAPP and the tendency of mature fibrils is to diminish over time. Studies in amyloid-beta (Aβ) present in Alzheimer's disease (AD) have been more extensive than those in human IAPP (hIAPP) and, taken in account that AD and T2D are linked to pathologies associated with protein aggregation, is tempting to assume the toxic oligomer hypothesis [73]. Over the last few years, a large number of data from *in vitro* studies in IAPP have emerged, however, the presence or absence of oligomers is still an open question. For that, specific antibodies were design to detect oligomers from a broad range of amyloid proteins, including IAPP. Recent publications have documented that A11 antibody was capable to recognize different types of toxic peptides linked to amyloid diseases, through the binding of native folded proteins that display anti-aggregation activity. Gurlo and colleagues were able to detect the presence of IAPP oligomers in insulin vesicles and mitochondrial membranes of hIAPP transgenic mice [74]. More recently, Soty et al. have demonstrated the formation of intracellular oligomers with an alteration in β-cell function [75].

According with the structural information available for IAPP fibrils and oligomers, it is clear that IAPP as an amyloid protein has shown to be toxic through similar mechanisms as other amyloid proteins. One of the most widely accepted mechanism of toxicity refers to membrane interaction which leads to cell membrane permeabilization or disruption [36]. Membranes are known to play a role in the misfolding of amyloid proteins. Its disruption can be affected by the toxic action of misfolded proteins and can be responsible for the rapid formation of oligomers or fibrils. Concerning hIAPP oligomers, the membrane leakage occurs via direct interaction and/or formation

of ion pores and depends on the lipid composition and on the lipid to peptide ratio as well as pH and ionic strength [76]. In addition, this pore forming capacity has been proposed for all amyloid proteins. In the case of fibril formation the damage in the membrane may happen through interaction of fibrils with specific channels located on the cell surface [39, 47]. Moreover, oligomers of hIAPP have been shown to increase inflammation in β-cells via the inflammasome [77].

Comparably, hIAPP can form proinflammatory oligomers and fibrils that contribute to islet inflammation by recruiting and activating macrophages *in vivo* [78]. Furthermore, Endoplasmic Reticulum (ER) stress has been proposed to be an important contributor to hIAPP induced β-cell death since exogenously added hIAPP has been confirmed to induce ER stress [74]. Some reports showed that ER stress-mediated apoptosis is exacerbated in rodent cells expressing amyloidogenic isoforms of hIAPP in β-cells, leading to a reduction of β-cell mass in hIAPP transgenic mice and rats [65, 79]. In addition, Casas et al. demonstrated that extracellular hIAPP aggregation is associated with ER stress responses in mouse β-cells, by an intracellular signaling that involves downstream inhibition of the ubiquitin-proteasome pathway, contributing to β-cell apoptosis [80-81]. Nevertheless, in a rat pancreatic β-cell line overexpressing hIAPP, the detection of toxic intracellular oligomers, which lead to defective insulin and IAPP secretion levels in response to glucose, did not change the expression of genes involved in ER stress [75]. Although the mechanism responsible for β-cell cytotoxicity during the process of hIAPP formation is still not well defined, a growing body of evidence firmly indicates that IAPP fibrils or oligomers have a crucial role in the progressive β-cell dysfunction in T2D.

2.4. MODELS OF HUMAN IAPP OVEREXPRESSION

2.4.1. The extracellular model: synthetic hIAPP

Several approaches have been applied to study amyloid toxicity *in vitro*; synthetic peptides, corresponding to either fragments or the whole protein, have been useful to attempt in defining the amyloidogenic pathology. Several studies have reported that amyloid peptide is proficient to induce cytotoxic cell death by external addition of synthetic hIAPP [69, 80, 82-84]. Although, the precise mechanism by which IAPP aggregates leads to β-cell death is still unknown, it has been

recognized that this aggregation is a concentration dependent of synthetic hIAPP *in vitro*. Bailey et al. suggested a progressive increase in cell toxicity according with the initial peptide concentration, as well as the time exposed for the process of IAPP fibrilization [71]. In addition, Casas et al. demonstrated that extracellular hIAPP (1-20 μM/l) aggregation is associated with ER stress responses in mouse β-cells, by an intracellular signaling that involves downstream inhibition of the ubiquitin-proteasome pathway, contributing to β-cell apoptosis (**Figure 13**) [80]. In line with these studies, evidences have demonstrated that in aqueous solution synthetic hIAPP spontaneously forms β-sheets and aggregates whereas synthetic rat IAPP does not [85-86] and the aggregation process seems to be extremely sensitive to amyloid concentrations [87].

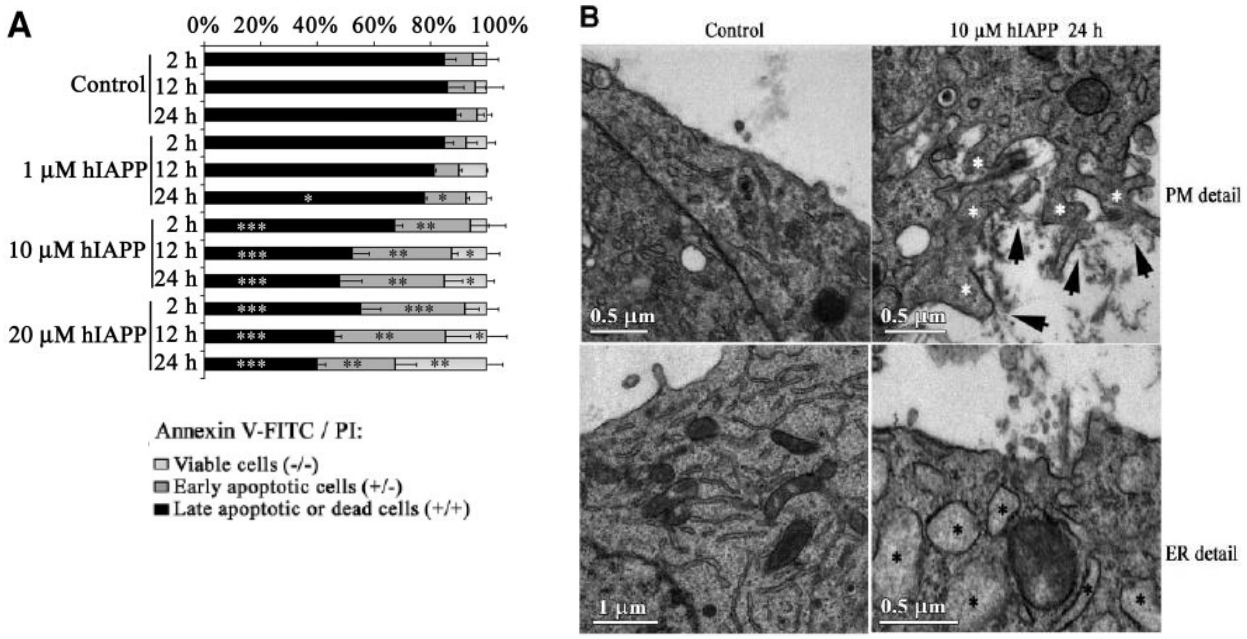


Figure 13. Toxic effect of extracellular hIAPP on MIN6 cells. Apoptosis assay on MIN6 cells treated with 1, 10, or 20 mol/l hIAPP compared with control. Cell staining with annexin V FITC and PI was analyzed by FACS **(A)**. Morphological analysis of MIN6 cells exposed to extracellular hIAPP compared with the control. *Upper panels* show how hIAPP aggregates were located close to the cell margins (arrows). The plasma membrane (PM) was irregular and showed prominent invaginations (asterisks). *Lower panels* show abnormal morphology of ER (asterisks) in treated MIN6 cells **(B)** [80].

2.4.2. The *in vitro* model

Although overexpression of human IAPP in transformed cells is known to cause IAPP, the mechanism by which amyloid oligomers and/or fibrils are formed is not completed understood. For that reason, many attempts to express this protein in various vectors and hosts have been designed. Eberhardt and colleagues were able to transfect fibroblast-like cell line (COS-1) cells with vectors expressing amyloidogenic IAPP; however, those cells containing amyloid fibrils were degenerated or dead when compared to rat IAPP overexpression [88]. Years later, the same group with the effort to understand the mechanism by which intracellular hIAPP causes cell death, demonstrated that in transfected COS-1 cells, the accumulation of hIAPP initiates a cascade of intracellular signaling events that triggers the apoptotic pathway [89]. However, this cell line was not expected to prevent such event due to the lack of the cellular machinery needed for the processing and trafficking of immature IAPP, such as secretory granules or prohormone convertases.

Recent studies have also reported successful cloning and expression of recombinant hIAPP in cultured mammalian cells. Several *in vitro* approaches allowed the successful expression, purification and characterization of the amyloidogenicity and cytotoxicity of the human mature IAPP in sufficient amounts using for example, the LacI-T7 RNA polymerase-based heterologous expression system for *Escherichia coli*. This pET *E. coli* expression system has been shown to remove potential toxic proteins and, at the same time generate high levels of recombinant proteins [90]. Likewise, other studies were capable to clone the hIAPP full-length peptide into, not only COS-1 cells, but also in rat insulinoma (RIN) and Chinese hamster ovary (CHO) cells [91]. Nevertheless, when studies were performed in INS1E cells, a β-cell line with all the equipment for the processing and regulation of IAPP, the expression of hIAPP by adenovirus have not resulted in cell death unless hIAPP was high enough to cause impaired proIAPP processing (**Figure 14**) [92].

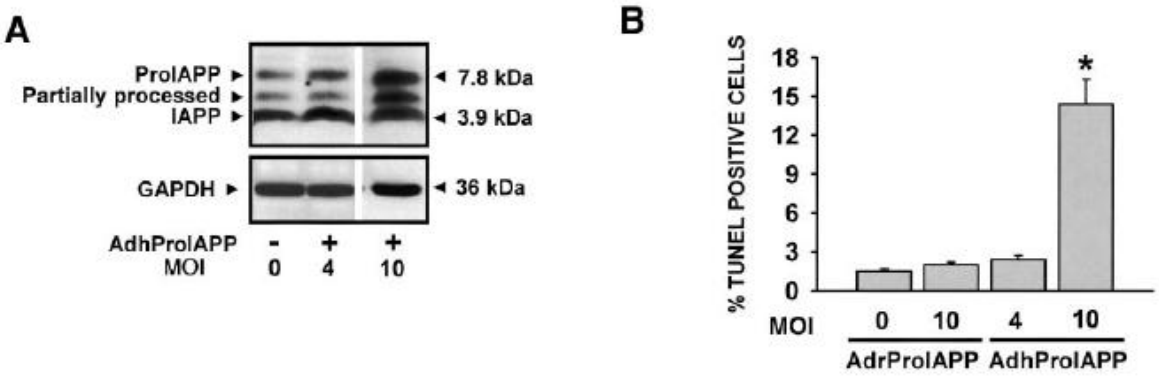
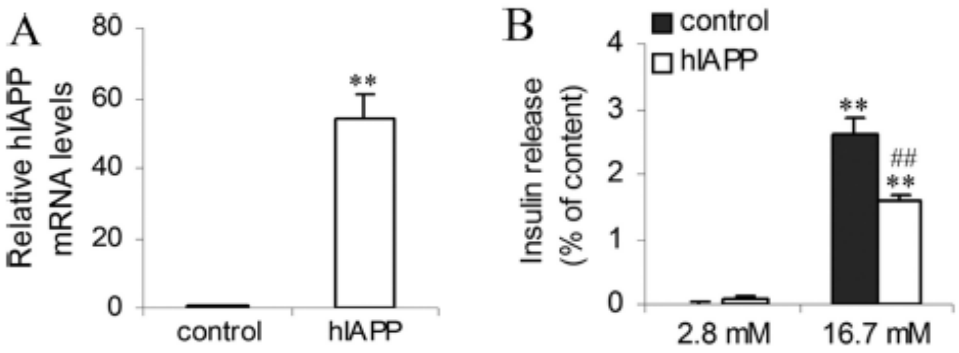


Figure 14. Impaired processing of human proIAPP is associated with increased cell death in INS1E cells. (A) INS1E cells were transduced with Ad-rProIAPP (MOI 10) or Ad-hProIAPP (MOI 4 or 10). **(B)** Increased number of apoptotic cells detected after 96h after transduction. Expression of human (but not rat) proIAPP at levels that resulted in impaired proIAPP processing (MOI 10) was associated with an increased cell death. [92].

Furthermore, our group has established an *in vitro* model in which INS1E cell line was stably transfected with human IAPP cDNA under the cytomegalovirus promoter (CMV). Under hIAPP overexpression, these cells showed intracellular oligomers and a strong alteration of glucose-stimulated insulin and IAPP secretion (**Figure 15**). Moreover, inhibition of insulin and IAPP secretion affected the activity of K_{ATP} channels leading to an increased mitochondrial metabolism in order to counteract the secretory defects of β-cells [75].



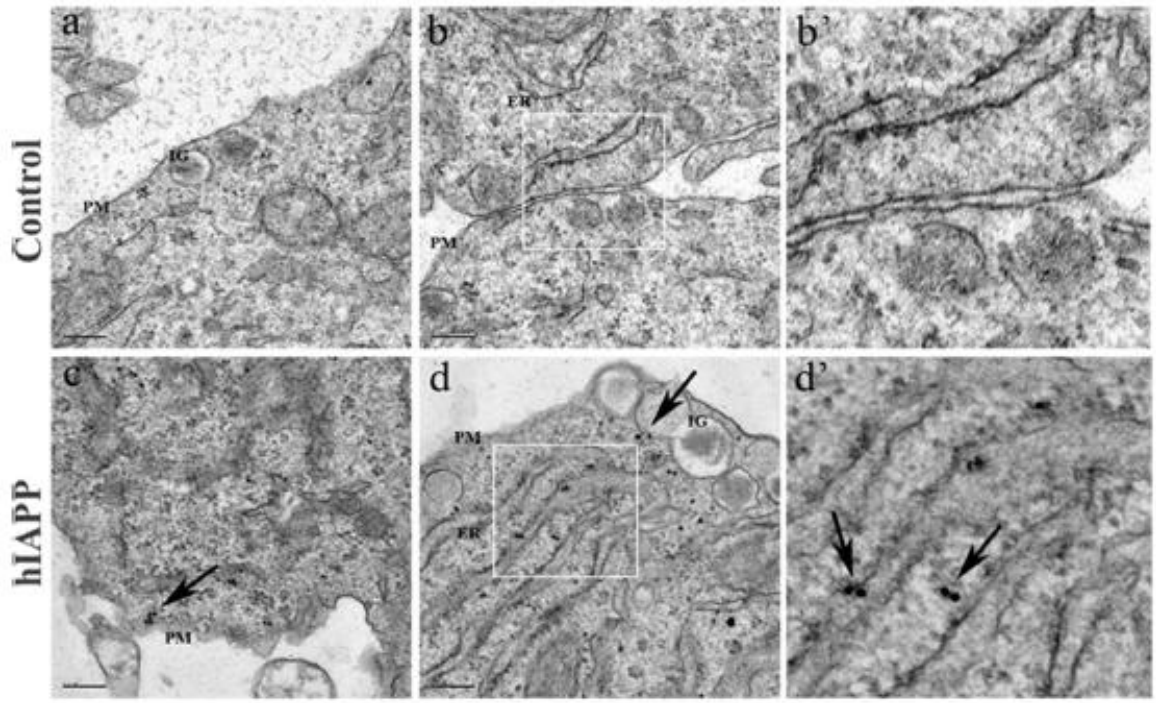


Figure 15. hIAPP-INS1E cells have impaired insulin secretory capacity due to intracellular oligomers. hIAPP mRNA levels **(A)** and Insulin secretion after glucose stimulation **(B)**. Immunogold labeling for oligomers in hIAPP-non-expressing cells (control) (*panels a*, *b*, and *b*') and hIAPP-expressing cells (hIAPP cells) (*panels c*, *d*, and *d*'). Oligomers were only detected in hIAPP cells in the ER (*arrows* in *panel d*'), in insulin granules (*IG*) (*arrow* in *panel d*), and associated to the plasma membrane (*PM*) (*arrow* in *panel c*) [75]**.**

2.4.3. The *in vivo* model: transgenic animal models with hIAPP overexpression

One of the most active research areas that have contributed substantially to our current understanding of the molecular basis in a multifactorial disease such as T2D is the creation and development of diverse animal models. Nowadays, *in vivo* studies of human pancreas morphology are not possible by obvious ethical considerations, and the collective human material comes from either autopsy or surgical resection from pancreatic cancer. It is interesting that apart from humans, the only species capable to spontaneously develop T2D are non-human primates and cats; nevertheless, besides the cost of working with such big species, these models not always progress towards T2D, making the use of these models not optimal for research [93]. Studies performed with rodent models of diabetes are then greatly useful and advantageous, especially regarding islet amyloidosis studies. Unlike the human islet amyloid polypeptide, the islet amyloid polypeptide from rodents is not amyloidogenic due to the proline substitutions in the IAPP 20-29 region. This lack of

amyloid development in these models makes impossible to assess the role of IAPP aggregation in the islet pathophysiology. Since only a limited number of species spontaneously form islet amyloid, several groups have developed transgenic mice strains choosing hIAPP as a model for islet amyloidogenesis (**Table 1**).

Nonetheless, some reports showed that the mere hIAPP overproduction did not lead to amyloid formation/deposition despite elevated plasma concentration of hIAPP. Thus, other factors beyond overexpression had to be involved in the mechanism of islet amyloid formation since these mice were normoglycemic and normoinsulinemic [94-96]. Islet amyloid was reported in transgenic mice fed a diet high in fat. Verchere and colleagues have shown that approximately 80% of male transgenic mice (>13 months of age) presented amyloid deposits and were always associated with severe hyperglycemia [56]. In the case of hemizygous transgenic mice for hIAPP, the treatment with growth hormone or dexamethasone contained small intra- and extracellular amyloid deposits [97].

Table 1. Transgenic rodent models for human islet amyloid polypeptide (hIAPP) [93].

Species	Transgene/ Background	Metabolic Phenotype	Islet Pathology	References (See Text)
Mouse	h-IAPP(hemizygous)/ FVB/N	Mice do not develop diabetes unless treated with growth hormone and dexamethasone.	1. Small intra- and extracellular amyloid deposits 2. Evidence of β-cell degeneration	Couce et al. 1996
Mouse	h-IAPP(homozygous)/ FVB/N	Mice spontaneously develop hyperglycemia (~11mM glucose) and diabetes by 10-14 wk of age.	1. Small intra- and extracellular amyloid deposits 2. Evidence of decreased β-cell mass	Janson et al. 1996
Mouse	h-IAPP(hemizygous)/ A ^{vy}/A	Progressive development of diabetes by ~ 15 wk of age in obese but not in lean transgenics	1. Large extracellular amyloid deposits 2. Decreased β-cell mass (~80%) 3. Increased β-cell apoptosis (~10 fold) 4. No change in β-cell neogenesis or replication	Butler et al. 2003b; Soeller et al. 1998
Mouse	h-IAPP(hemizygous)/ C57BL/6J	Mice do not spontaneously develop diabetes. Mice exhibit impaired insulin secretion in response to oral glucose load.	1. No reported presence of extracellular amyloid deposits 2. Electron microscopy reveals aggregation of amyloid fibrils in β-cell secretory granules 3. No change in β-cell mass	Ahren et al. 1998; de Koning et al. 1994; Hoppener et al. 1999
Mouse	h-IAPP(hemizygous)/ C57BL/6J	Mice do not spontaneously develop diabetes and display normal glucose tolerance in vivo. Mice show evidence of impaired glucose-induced insulin secretion in isolated islets.	1. No extracellular amyloid deposits 2. Reported formation of amyloid fibrils in β-cell secretory granules 3. No change in β-cell mass	Tokuyama et al. 1997; Yagui et al. 1995
Mouse	h-IAPP(hemizygous)/ C57BL/6 × DBA	Mice do not spontaneously develop diabetes, however, tend to demonstrate impaired glucose tolerance in response to IPGTT due to diminished insulin secretion.	1. Presence of extracellular amyloid deposits 2. Evidence for decrease in β-cell mass (~15%)	Hull et al. 2003; Hull et al. 2005; Wang et al. 2001
Mouse	h-IAPP(hemizygous)/ ob/+	Mice spontaneously develop diabetes and stay hyperglycemic (~15-20 mM glucose) during their life span compared with nontransgenic ob/ob mice.	1. Presence of extensive extracellular amyloid deposits. 2. Substantial decrease in β-cell mass	Hoppener et al. 1999
Rat	h-IAPP(homozygous)/ CD	Sudden onset of diabetes with the first 2 mo of age	1. No extracellular amyloid deposition. 2. Rapid decline in β-cell mass	Butler et al. 2004
Rat	h-IAPP(hemizygous)/ CD	Gradual onset of diabetes by midlife ~5-10 mo of age	1. Presence of extensive extracellular amyloid deposits. 2. Decreased in β-cell mass (~50-80%) 3. Increased β-cell apoptosis (~10-fold) 4. Increased β-cell replication, with no change in neogenesis	Butler et al. 2004

Another strategy used to overexpress hIAPP was to cross-breed hIAPP mice onto a mouse with obese background (*ob/ob*) [98-99] (**Figure 16**) or obese Agouti viable yellow (*Avy/Agouti*) [100]. These mice developed amyloid formation and loss of β-cells, which was associated with progression of diabetes. Moreover, it was found that female transgenic mice do not increased the

occurrence of amyloid when oophorectomized, suggesting a protective role of ovarian hormones in islet amyloidosis. In recent years, Butler's laboratory showed that transgenic β-cell expression of human proIAPP in rats (HIP rats) that are hemozygous for hIAPP develop diabetes within 5-10 months, together with the presence of extracellular amyloid, decreased β-cell mass and increased β-cell apoptosis [101]. The loss of approximately 60% in β-cell mass at the onset of diabetes demonstrated that HIP rat, is similar to the 65% β-cell deficit observed in humans with T2D [31].

With a variety of transgenic hIAPP models it has been possible to clearly highlight that islet amyloid formation is a complex event associated with a great number of factors considered important in the pathogenesis of these deposits.

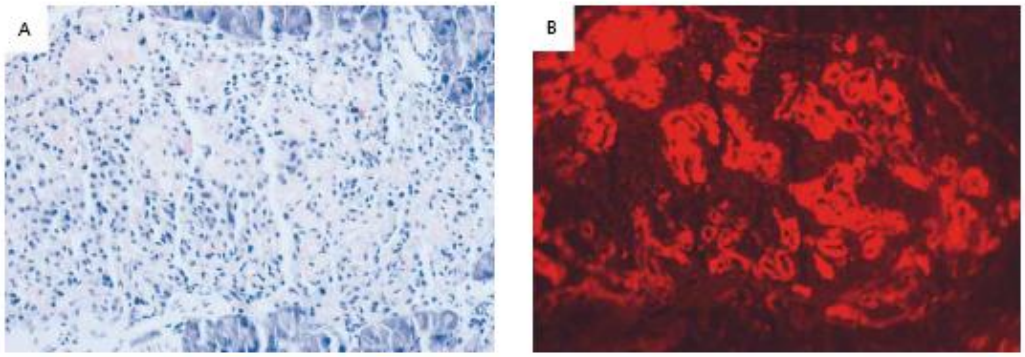


Figure 16. Histochemical findings in pancreatic specimens from a 17-month-old transgenic *ob/ob* mouse that produced human islet amyloid polypeptide. In 80 percent of the islets of Langerhans from the mouse, Congo red staining revealed extensive, pink amyloid deposits under white-light microscopy (**A**). Red autofluorescence of amyloid deposits in the same islets is evident under ultraviolet-light microscopy (**B**) [99].

3. THE ENDOPLASMIC RETICULUM

3.1. PHYSIOLOGICAL ROLE

In Eukaryotic cells, the Endoplasmic Reticulum (ER) consists in a vast membranous organelle that forms an interconnected network focused first and foremost on the secretory pathway. The translation of the vast majority of secreted and transmembrane proteins, are

performed by ribosomes on the cytosolic surface of the ER, where the newly translated unfolded polypeptide chains are translocated inside the ER lumen, and where is possible the interaction between chaperones and folding enzymes in a highly oxidizing and Ca^{2+} store environment. The ER is considered as the main intracellular reservoir for Ca^{2+} and its concentration is maintained higher inside than in the cytosol. This regulation is produced by the sarco(endo)plasmic reticulum Ca^{2+} ATPase (SERCA) which pumps Ca^{2+} into the ER lumen and is a key component for cellular signaling, adaptation and survival. Moreover, the ER plays a central role in free fatty acid esterification and steroid production and storage [102]. Dynamic interactions and crosstalk between ER and other organelles has been reported. As an example, the secretory and membrane proteins that are transported from ER to Golgi in vesicles [103]. Furthermore, a growing number of studies point to the role of the ER in the sensing and transduction of apoptotic signals [104]. The improved understanding of the molecular basis for ER processing activities could reveal the role of the ER in the development of a variety of diseases including T2D.

3.2. PROTEIN FOLDING AND/OR MISFOLDING IN THE ER. THE ROLE OF CHAPERONES

Protein folding begins as the nascent polypeptide chain is co-translationally translocated through the ER membrane into the ER lumen. This process is possible due to the binding of the ribosome to the Sec61p complex, a heterotrimeric protein complex in the ER membrane, which guarantees that the elongating nascent chain is transferred from the cytosol directly into the ER lumen [105]. The unique environment of the ER lumen allows for both oxidative protein folding as well as post-translational modification such as, glycosylation and disulphide bond formation, and accounts for approximately one third of all proteins in a eukaryotic cell [106]. Although most of small proteins are thought to fold rapidly, several proteins required long periods before they attain their final three-dimensional structure. Since ER is mainly associated with protein synthesis, if the protein is not properly folded/matured, it will remain in the ER and will eventually be degraded without reaching its normal cellular site of action [107] (**Figure 17**).

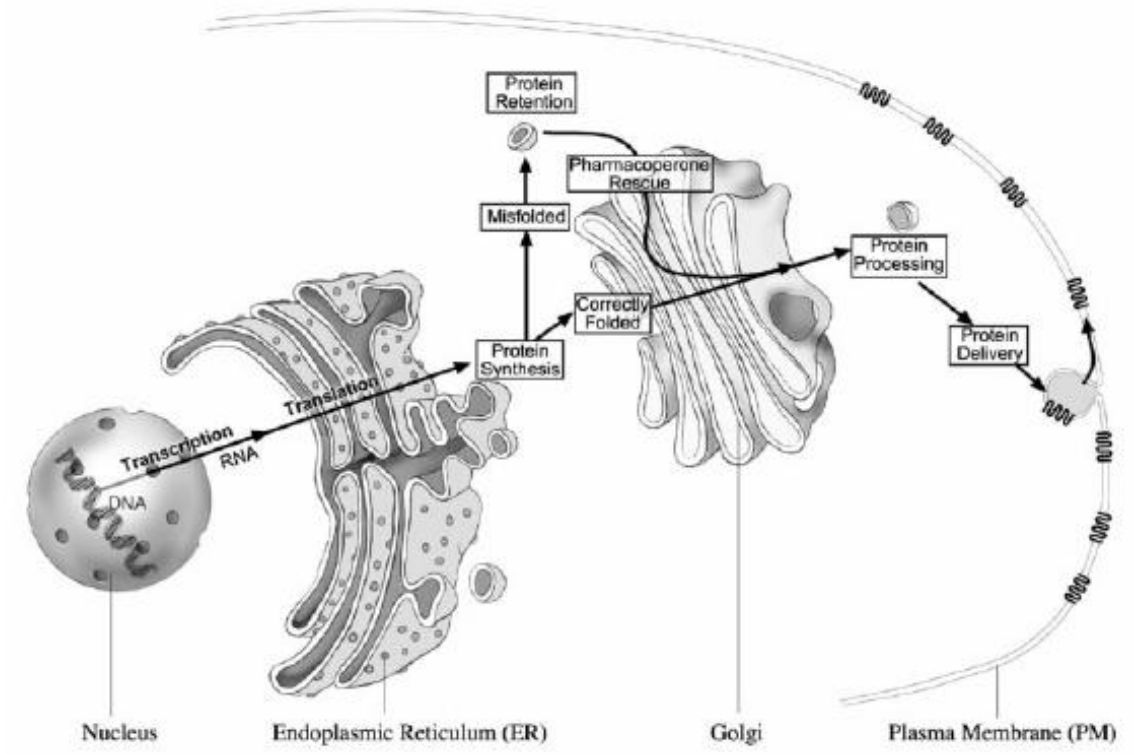


Figure 17. Cellular sites associated with protein synthesis [108].

This sophisticated supervision carried by the ER is regulated by a sensitive quality control (QC) system that can discriminate between the proper folded proteins from the misfolded ones. The QC system operates at several levels and by multiple mechanisms [109] (**Figure 18**). One of them is the folding chaperone system consisting on a considerable number of proteins, termed chaperones, that have the capacity to recognize properties common to non-native proteins such as exposed hydrophobic areas and in most cases through the expenditure of ATP [110]. The ER contains three groups of molecular chaperones and folding enzymes: chaperones of the heat shock protein family, such as BiP, chaperone lectins including, calnexin and calreticulin, and thiol oxidoreductases of PDI family. Most of these chaperones have an important role in the correctly folding and assembly of secretory proteins, oligomerization and post-translational modifications [111]. Recent evidences that chaperones are required to prevent misfolding proteins connect with the fact that the concentrations of many of these factors are significantly increased during cellular stress [112].

The BiP (immunoglobulin heavy-chain-binding protein) also known as GRP78 (78 kDa glucose-regulated protein), is one of the most abundant ER chaperone and has been extensively implicated in the mechanism of protein folding. Besides assisting in *de novo* folding, BiP has various other functions, including protein trafficking, mediates retention of proteins in the ER, prevention of protein aggregation, and a role in the translocation of newly synthesized proteins across the ER membrane [112].

The formation of disulfide bonds is also a critical step in the maturation of the majority of the proteins that traffic through the ER. The most studied mammalian enzyme responsible for this process is the protein disulfide isomerase (PDI). PDI is considered as a multifunctional protein that can act as both an oxidoreductase as well as a chaperone. Regarding its molecular chaperone activity, PDI avoid the aggregation of misfolded proteins that do not contain any disulfide bond [113]. The other component of the QC system is the ubiquitin-proteosome system (UPS) in which irreparably damaged proteins are identified and sorted for degradation. This system is responsible for the clearance of intracellular misfolded and aggregated proteins.

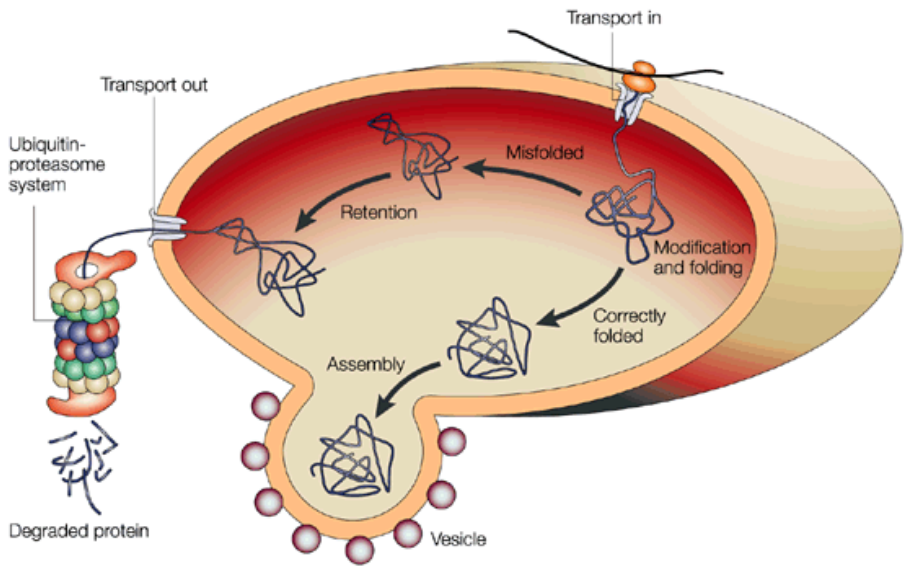


Figure 18. Regulation of protein folding in the ER [114].

Folding and/or misfolding are crucial ways of regulating biological activity and targeting proteins to different cellular locations. Moreover, specific types of cellular activities such as, translocation through membranes, trafficking, secretion, and regulation of the cell cycle among others, depend directly on the folding and unfolding events. Many stimuli can disrupt this process and its failure can give rise to the malfunctioning of living systems leading to the development of an increasing number of disorders, including Parkinson, Alzheimer, Huntington and T2D [115].

3.3. ER STRESS AND THE UNFOLDED PROTEIN RESPONSE

As mentioned above, proteins are properly folded and assembled with the assistance of molecular chaperones and folding enzymes. Therefore, an acute increase in the translation of secretory proteins could represent a major problem for the cell due to a probable production of unfolded or misfolded proteins. However, this situation may turn out to be more critical if there is an alteration in the ER homeostasis due to altered glycosylation, energy depletion, ER Ca^{2+} depletion, oxidative stress, energy deprivation, metabolic challenge and inflammatory stimuli [116]. These physiological and pathological conditions may interfere with protein maturation and trafficking processes leading to the accumulation of unfolded and/or misfolded proteins.

Over the last decade, a complex homeostatic mechanism known as the unfolded protein response (UPR) has evolved linking the load of newly synthesized proteins with the capacity of the ER to mature them. The UPR works as a multifaceted strategy to protect the integrity of the ER and the associated functionality of the secretory pathway. In mammalian cells, the first response consists in attenuating the translation of most peptides, followed by an induction of ER chaperone translation that promotes the correct protein folding [104] and finally the activation of the ER-associated degradation (ERAD), in which misfolded proteins are re-trotranslocated from the ER lumen to the cytosol and degraded by the ubiquitin-proteasome [104, 117]. Conversely, if this mechanism of adaptation and survival fails to relieve ER stress, a continued accumulation of misfolded proteins takes place within the ER and consequently UPR will generate pro-apoptotic signals to eliminate the diseased cell [104].

In mammals, the UPR consists of three main classes of sensors of ER stress: inositol-requiring enzyme 1 (IRE1), double-stranded RNA-activated 9 protein kinase (PKR)-like ER kinase (PERK) and activating transcription factor 6 (ATF6) (**Figure 19**).

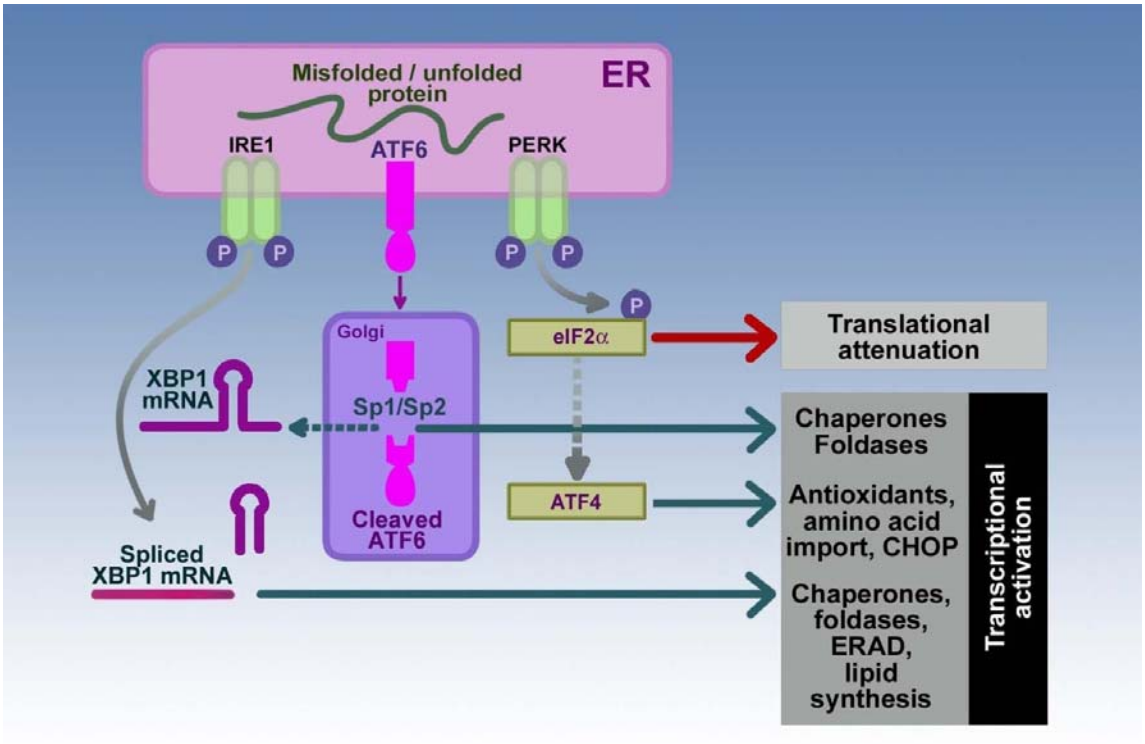


Figure 19. Branches of the UPR signaling pathway. The three sensor-transducers of the UPR are inositol-requiring protein-1 (IRE1), activating transcription factor-6 (ATF6), and protein kinase RNA (PKR)-like ER kinase (PERK). These sensor-transducers determine the state of unfolded proteins in the ER lumen [118].

Despite the difference, each of these signaling pathways, on the other hand, activate transcription factors that mediate the induction of several ER stress genes. The IRE1, PERK, and ATF6 signaling pathways of the UPR that are crucial in cell survival responses are examined in detail below.

3.3.1. The IRE1 signaling pathway

Inositol-requiring enzyme 1 (IRE1) is considered the oldest branch of the UPR and was the first component identified as the ER stress sensor in yeast [119]. IRE1 is a type 1 ER-transmembrane protein that contains both, a serine/threonine kinase and cytoplasm RNase domains. Considered a central regulator of ER stress signaling, plays an important function in the regulation of protein biosynthesis. Two mammalian homologues of yeast IRE1 have been identified: IRE1α, which is conserved in all eukaryotic cells and expressed ubiquitously [119]; and IRE1β, which is expressed only in intestinal epithelial cells [120].

Under non-stress conditions, IRE1 remains in an inactive monomeric form. Upon accumulation of unfolded proteins in the ER lumen, IRE1 is activated, and released by the chaperone BiP from its regulatory domains leading to dimerization and transphosphorylation [121]. Transphosphorylation of the kinase domain of IRE1 activates its RNase activity, whereby IRE1 initiates precise endonucleolytic cleavage of an *mRNA* encoding a transcription factor called X-box binding protein-1 (XBP1). The RNase activity of IRE1 initiates splicing of a 26-base intron from the XBP1 mRNA leading to an activated version of the transcription factor spliced XBP1 (sXBP1). Once translocated to the nucleus, sXBP1 initiates several transcriptional programs that up-regulate UPR-associated genes including many genes responsible for ER associated degradation (ERAD) and some chaperones [122] (**Figure 20**).

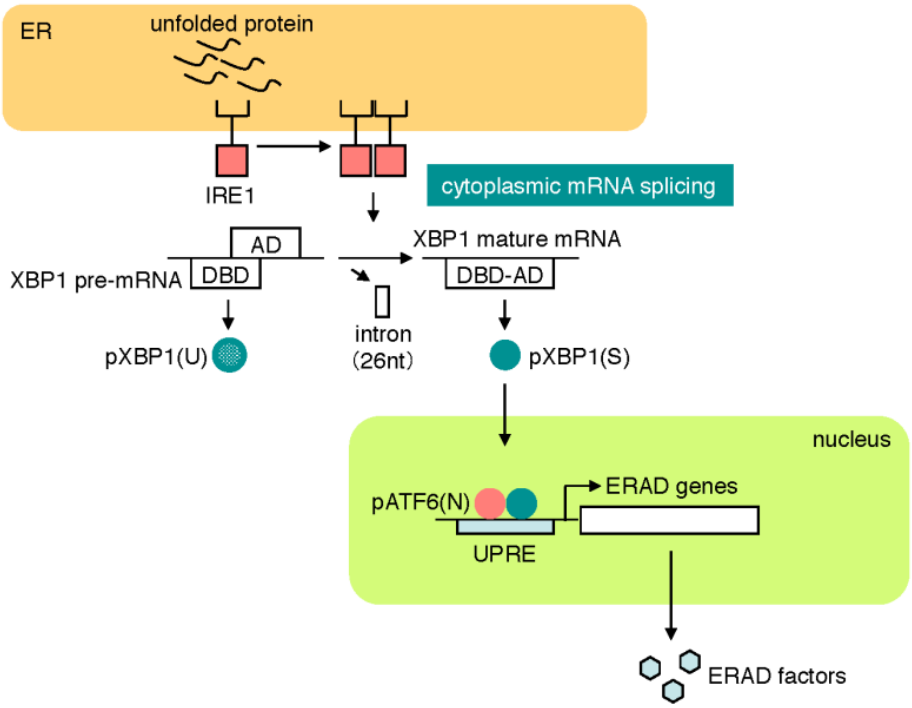


Figure 20. The IRE1 pathway. In normal conditions IRE1 is an inactive monomer, whereas IRE1 forms an active oligomer in response to ER stress. Activated IRE1 converts unspliced *XBP1* mRNA to mature mRNA by cytoplasmatic mRNA splicing. The active transcription factor pXBP1(S) is translated and activates the transcription of ERAD genes through binding to the enhancer UPRE [123].

3.3.2. The PERK signaling pathway

A reduction in protein translation and in ER workload is the first response to counteract ER stress. This is mediated by the PERK signaling pathway. Once chaperone protein BiP releases from its interaction with PERK's luminal domain, PERK is able to dimerize, promoting autophosphorylation and activation [120]. Once activated, PERK phosphorylates serine-51 of eukaryotic initiation factor 2α (eIF2α), its only recognized target. When phosphorylated, eIF2α is unable to efficiently initiate translation, leading to a rapidly reduction in the number of proteins entering the already overwhelmed ER [124]. However, some selected proteins such as the activating transcription factor 4 (ATF4) mRNA are preferentially translated and modulates the expression of some other genes such as activating transcription factor 3 (ATF3) and C/EBP homologous protein (CHOP). CHOP acts by inducing the expression of the growth-arrest and DNA-damage-inducible protein 34 (GADD34) which acts to dephosphorylate eIF2α contributing to reactivation of protein translation [125] (**Figure 21**).

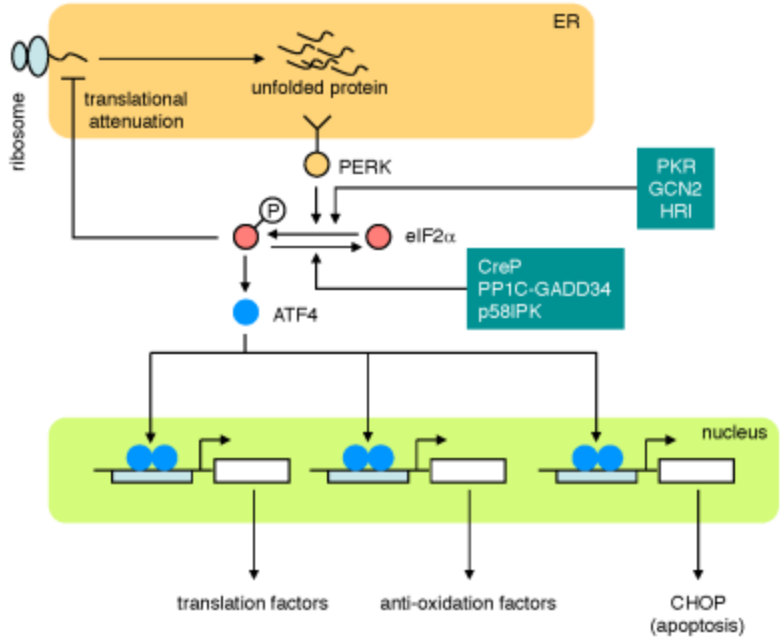


Figure 21. The PERK pathway. When PERK detects unfolded proteins in the ER, phosphorylates eIF2α, resulting in translational attenuation and translational induction of ATF4. ATF4 activates the transcription of target genes encoding translation factors, anti-oxidation factors and the transcription factor CHOP [123].

On the other hand, translation attenuation is thought to be an adaptive response that helps cells survive ER stress, protecting from ER stress-induced apoptosis [126]. It has been shown that subsequent translation attenuation is responsible for the activation of the nuclear factor kappa B (NF-κB) as a result of inhibited enzyme IκB kinase (IKK) translation, leading to cell protective gene expression changes [127-128]. Therefore, optimal function and survival of diverse secretory cells depends on translational regulation via PERK/eIF2α branch of the UPR.

3.3.3. The ATF6 signaling pathway

A major mediator of transcriptional induction by ER stress is the basic leucine zipper domain transcription factor ATF6. This protein is regulated not only by interaction with BiP, but also by intra- and intermolecular disulfide bridges which are thought to keep ATF6 inactive [129-130]. In response to ER stress, BiP is released from ATF6 and disulfide bonds are reduced, which stimulates its own translocation to the Golgi, where site-1 protease (S1P) and site-2 proteases (S2P) are

cleaved and conducted to the nucleus to target transcription chaperones, elements of the ER-associated degradation pathway (ERAD), and the up-regulation of XBP1 (**Figure 22**). Under chronic ER stress, ATF6 attempts to suppress the apoptotic UPR signaling cascade by up-regulation of the PERK and IRE1 pathways.

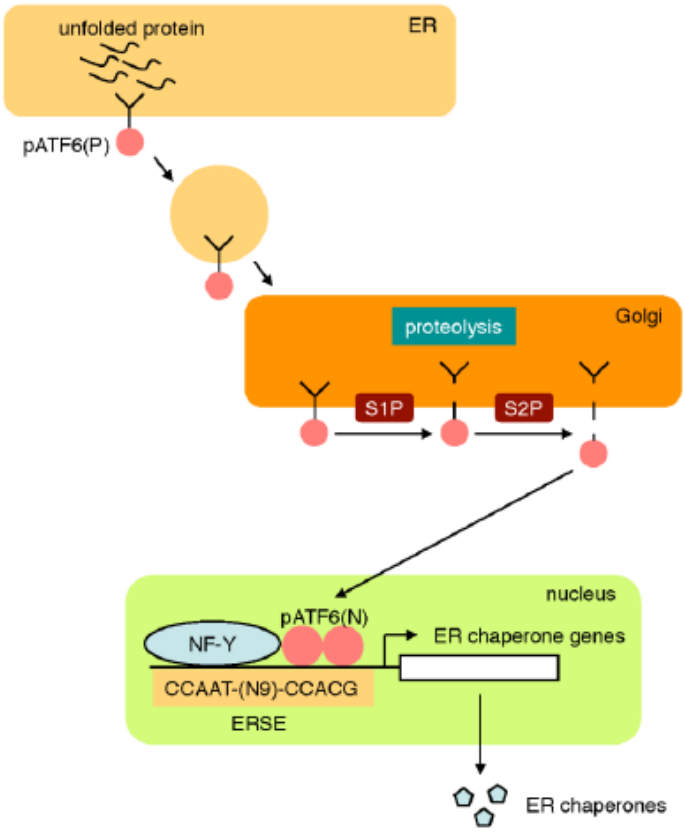


Figure 22. The ATF6 pathway. pATF6 is transported to the Golgi in response to ER stress, where is cleaved by S1P and S2P proteases, resulting in release of the cytoplasmatic portion pATF6(N) from the ER membrane. pATF6(N) translocates into the nucleus and activates transcription of ER chaperone genes through binding to the cis-acting enhancer ERSE [123].

3.4. LINKERS OF STRESS AND APOPTOSIS IN T2D

A growing number of studies implicate ER stress in the loss and death of β-cells during the evolution of T2D [131-132]. The ER is considered a vital organelle for protein synthesis and maturation, quality control, and secretion [133-134]; however, these processes require a stable

environment for balancing ER protein load and ER folding capacity. Nevertheless, it is known that several physiological, environmental and genetic factors can provoke alterations in ER homeostasis leading to a state of stress. Indeed, the evidences suggest that a continuously increase in insulin biosynthesis observed in T2D patients, might overwhelm the folding capacity of the ER leading to a state of chronic stress. As a consequence and as described above, the three UPR signaling pathways are triggered in order to mitigate ER stress, maintaining β-cell function and promoting β-cell survival through mechanisms of attenuation of protein translation, up-regulation of chaperones and increasing folding capacity. In general, cells have the capacity to adapt to substantial ER stress, not only by activation of UPR pathway, but also through ERAD pathway, in which damage or unfolded proteins are selectively targeted and ubiquitinated for degradation by the proteasome to prevent misfolding-induced toxicity [135]. Though if ER stress is too severe and long-lasting, the UPR-mediated efforts ultimately fail and the apoptotic pathway is activated in order to protect the organism by eliminating the damaged cells. Accumulating evidences points to ER as the main organelle responsible for sensing and transducing apoptotic signals.

At least three parallel pathways are involved in the stress-mediated apoptosis: activation of CHOP; activation of the IRE1-JNK pathway; and activation of caspase-12 [136-137] (**Figure 23**). The first apoptosis pathway is the transcriptional induction of the gene for CHOP also known as GADD153, and has been recognized as a key mediator of apoptosis in ER stress. Its expression is barely detected under physiological conditions, but is markedly perceived in response to ER stress. Additional mechanisms of apoptotic induction have been associated with particular branches of the ER stress pathway. The second in ER stress is cJUN NH_2-terminal kinase (JNK) pathway which is mediated by formation of the inositol requiring 1 (Ire1)-TNF receptor-associated factor 2 (TRAF2)-apoptosis signal-regulating kinase1 (ASK1) complex. Under chronic ER stress, IRE1 becomes hyperactivated and phosphorylates the pro-apoptotic factor JNK. The third pathway is related with activation of ER-associated caspase-12. Procaspase-12 is localized in the ER membranes and undergoes cleavage during ER stress in murine cells, promoting the downstream cleavage of caspase-3, the last effector caspase of the apoptotic cascade [138-139]. Together, cell survival and cell death factors represent key opposing forces underlying stress response.

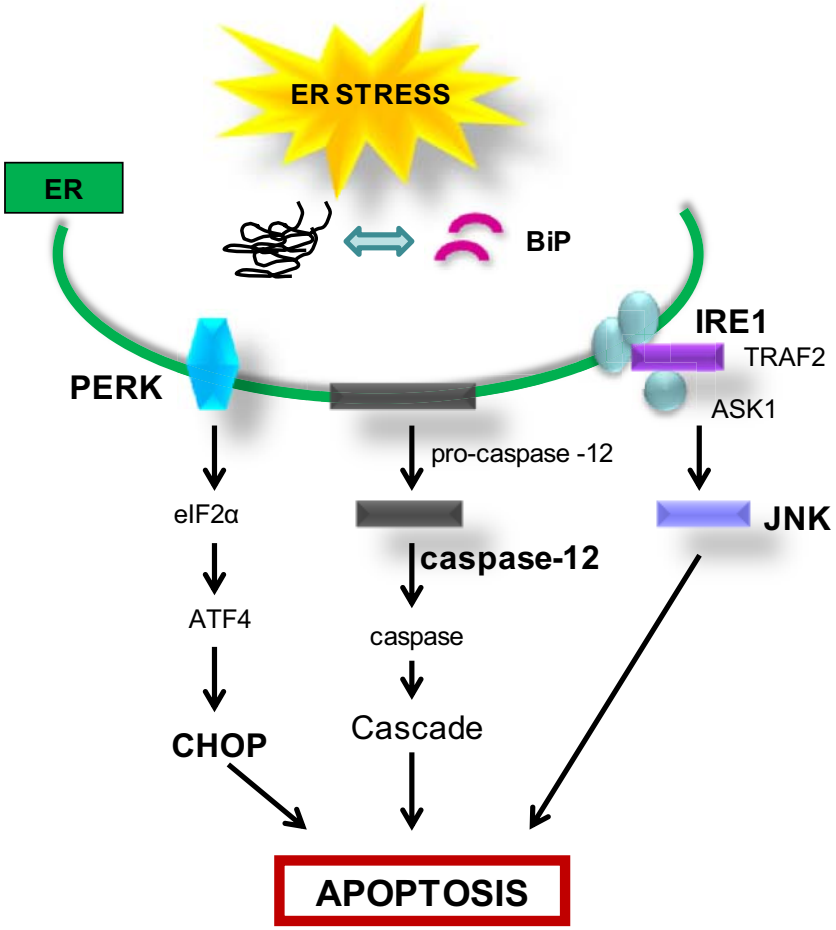


Figure 23. Pathways of ER stress-induced apoptosis. (1) The pro-apoptotic pathway of CHOP transcription factor which is mainly induced via PERK/eIF2α, (2) IRE1-mediated activation of ASK1/JNK, (3) activation of the ER localized caspase-12. These three pathways end in caspase cascade activation, the execution phase of ER stress-induced apoptosis (Adapted from van der Kallen et al., 2009) [140].

3.5. PATHOPHYSIOLOGICAL ER STRESS INDUCERS IN β-CELLS

3.5.1. The effect of hyperglycemia and free fatty acids

Chronic hyperglycemia and chronic elevation of free fatty acids (FFA) associated with obesity and T2D are the two major factors contributing to β-cell dysfunction and loss of β-cell mass [141]. Chronic hyperglycemia is a condition in which an excessive amount of glucose circulates in the blood plasma and is one of the classical manifestations of T2D. It is known that β-cell has the capacity to adapt to hyperglycemia by expansion of cell mass and by metabolic changes. However, long-term hyperglycemia is considered detrimental for the development of T2D and is associated

with alterations in β-cell mass and function [142], leading to cell exhaustion and induced by the toxic effects of glucose. In addition, it has been proposed that induced ER stress in β-cell is due to an excessive stimulation of insulin synthesis, leading to an overload of the ER and/or perturbation of Ca^{2+} homeostasis which is a result of an overstimulation of the ATP-dependent potassium channels (K_{ATP}) [143]. It was demonstrated that chronic exposure of glucose to rat insulinoma cells and mice islets, induces the activation of IRE1α and the alternative splicing of XBP1 [144]. The activation of this pathway is associated with moderate stimulation of JNK which could increase the propensity of the β-cells to apoptosis [145]. PERK pathway is also implicated in conditions of glucotoxicity. Studies have confirmed that rat islets exposure to high glucose increase the expression of downstream targets of PERK, such as CHOP and ATF3 [146]. Staining of these markers was shown in pancreatic sections from patients with T2D. Similarly, in *db/db* mice, phosphorylation of eIF2α is augmented together with CHOP and ATF4 expression. [147].

FFAs are also another important energy source for the body. They are implicated in mammalian homeostasis, particularly in the formation of biological membranes and as fuel for energy production in tissues such as the heart and skeletal muscle. Evidences have shown that saturated fatty acids such as palmitate are highly toxic to β-cell, in contrast unsaturated fatty acids such as oleate shown to have protective effect [148]. The contribution of high levels of saturated FFA are proposed to trigger ER stress signalling in pancreatic β-cells *in vitro* and in β-cells of T2D patients [149-150]. A recent study proposed the process of protein palmitoylation that occurs by addition of 16-carbon saturated palmitic group on cysteine residues. These mutations in proteins alter signal recognition or folding, inducing ER stress [151]. In addition, palmitate is capable to activate all the three branches of UPR [152] (**Figure 24**). Additionally, it has been demonstrating not only FFA could induce ER stress through enhancing Ca^{2+} influx but also, activate calpain-2 to induce CHOP expression and β-cell apoptosis [153]. Besides, the depletion of Ca^{2+} by palmitate delays ER-to-Golgi protein trafficking, disturbing in this manner, the build-up of proteins and promoting an overload within the ER [154].

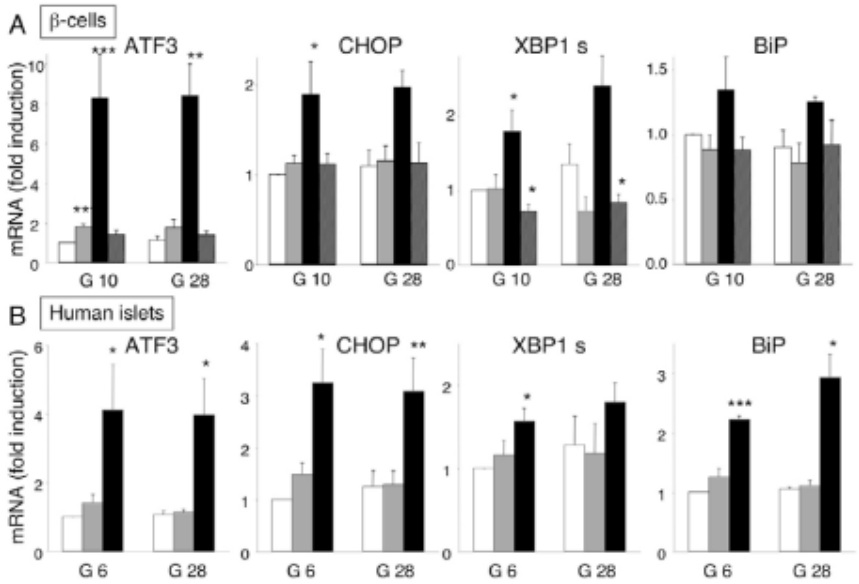


Figure 24. FFA activates ER stress signaling in rat primary β-cells and human islets. (**A**) FACS-purified rat β-cells were cultured for 24 hours in the presence of oleate (gray bars), palmitate (black bars) or oleate plus palmitate (hatched bars) at glucose concentrations of 10 and 28 mM. (**B**) Human islets were cultured for 48 hours in the absence (white bars) or presence of oleate (gray bars) or palmitate (black bars) at glucose concentrations of 6.1 and 28 mM.

3.5.2. The effect of glucolipotoxicity

In the process of glucolipotoxicity, there is a synergistic effect of high glucose and saturated FFAs in the impairment of β-cell function and survival. Although the exact mechanisms of glucolipotoxicity are still unclear, several studies point to the presence of hyperglycemia as a main cause for the toxic actions of FFAs [155]. One of the proposed mechanisms is the decrease of fat oxidation and consequently the detoxification of fat, while at the same time the conversion of FFAs into toxic complex lipids. This is known as the Malonyl-CoA hypothesis, in which Malonyl-CoA inhibits carnitine-palmitoyl-tranferase-1 (CPT-1), the enzyme responsible for transport of FFA into the mitochondria for β-oxidation, leading to the accumulation of long chain fatty-CoA in the cytosol, which is proposed to exert deleterious effects such as insulin resistance in muscle, impaired glucose induced insulin secretion or β-cell apoptosis [141]. Studies also implicate the low activation of AMP-activated protein kinase (AMPK) in this process. Indeed, AMPK acts as a “fuel sensor” and is capable to make changes in gene expression leading to enhanced lipogenesis through the transcription factor sterol-regulatory-element-binding-protein-1c (SREBP1c) [156-157]. This study was supported

by the finding that the treatment with an AMPK activator, such as Metformin, protected β-cell from glucolipotoxicity [158]. As mentioned above, insulin secretion is also affected due to alterations in the pancreatic and duodenal homeobox 1 (Pdx1) and the transcription factor MafA binding to insulin promoter [159]. Another possible mechanism regarding inhibition of insulin secretion, implicates the increased expression of the uncoupling protein-2 (UCP2) as the main responsible for the augmented levels of ROS production [160-161].

A large number of studies have been employed to identify the cellular and molecular basis of glucolipotoxicity and to show how harmful could be the combination of hyperglycemia and FFA for β-cells. Recently, considerable evidences have been provided in support of a role for the UPR and the loss of ER homeostasis when β-cells are exposed to glucolipotoxicity. Tanabe et al. demonstrated that MIN6 cells and primary islets treated with glucose together with FFA activate ER stress markers such as ATF3, along with the recruitment of SREBP1 to the nucleus and the impairment of FFA oxidation. The excess of unmetabolized FFA would lead to a division in ER membranes conducting to ER stress. In addition, both SREBP1 and ATF3 were able to inhibit IRS2 causing a negative effect on insulin signaling [162] (**Figure 25**).

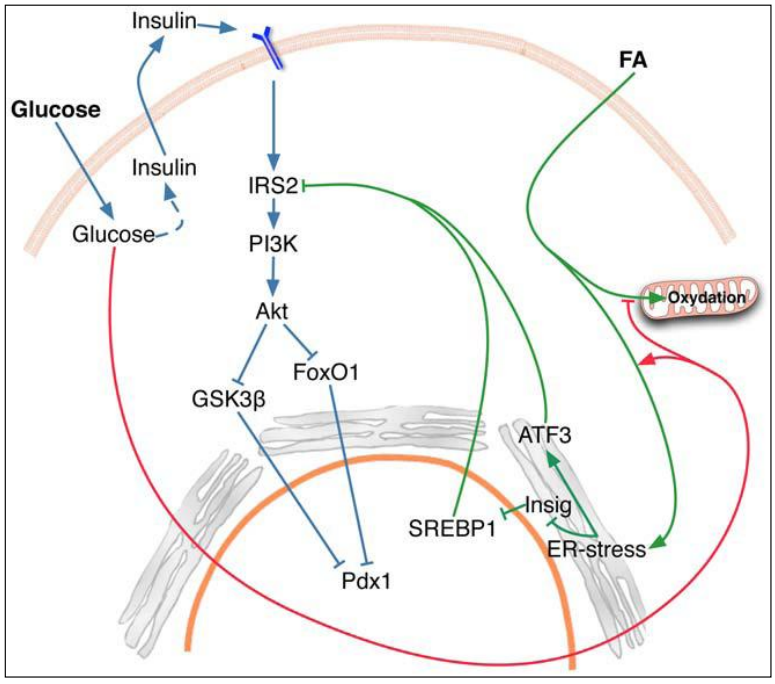


Figure 25. Key steps involved in ''glucolipotoxicity'' of β-cells [162].

Another study demonstrated that glucose amplifies palmitate-induced ER stress by increasing IRE1α and CHOP protein levels activating the JNK pathway, leading to increased β-cell apoptosis via activation of mTORC1 [145] (**Figure 26**). Although the exact mechanisms are uncertain, it is clear that the combination of elevated FFA and hyperglycemia, as occurs in insulin resistant individuals, synergize to produce an eventual destruction of β-cells.

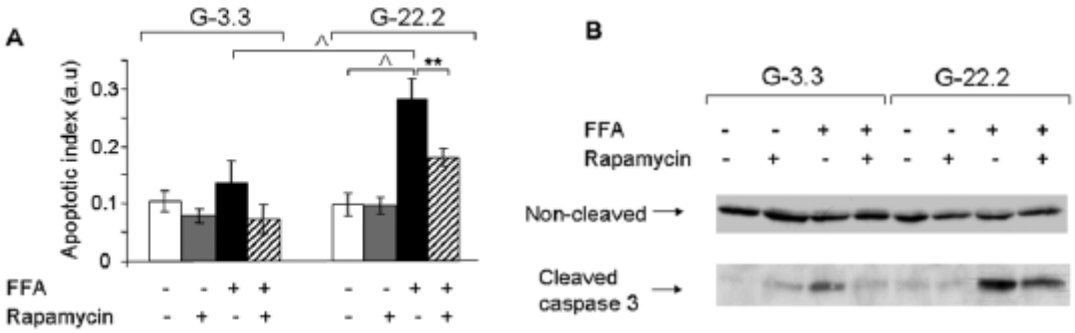


Figure 26. Glucose amplifies palmitate β-cell apoptosis via mTORC1 stimulation. INS1E cells were incubated at 3.3 and 22.2 mmol/l with 0.5% BSA with and without 0.5 mmol/l and 50 nmol/l rapamycin for 16 h. Apoptosis was assessed using the Cell Death ELISAPLUS assay (A) and by Western blot for cleaved caspase 3 (B) [145].

4. MOLECULAR AND CHEMICAL CHAPERONES: THE EMERGING ROLE IN T2D

An increasing body of evidence suggests that chaperones exert important protective effects in the decrease of ER stress, protein aggregation and the pathophysiology of amyloid deposition [112, 163-164]. Chaperones within the ER are coordinately upregulated at steady levels during ER stress defense maintaining the ER in unstressed conditions. Moreover, the ER has been implicated in several pathologies including cancer, neurodegenerative diseases, obesity, and diabetes, leading us to assume that promoting ER folding capacity through chaperones could be a novel target in therapeutic approach [165].

Molecular Chaperones

In recent years, several studies have provided evidences that the use of molecular chaperones could be a promising strategy for treating ER stress-related human diseases such as T2D. It has been shown that overexpression of certain particular ER chaperones can protect cells against cell death caused by disturbances of ER homeostasis [164, 166-168]. Chaperone BiP has been extensively implicated in the pathogenesis of misfolded diseases, including T2D. Of interest, transgenic mice overexpressing the molecular chaperone BiP specifically in β-cells are protected against the injury of obesity-induced T2D, maintaining β-cell function and improving glucose homeostasis [167]. Overexpression of BiP attenuates fatty acid-induced ER stress and apoptosis in hepatocytes [168]. These findings suggest that increasing chaperone expression can be an effective strategy for delaying or even prevent cytotoxicity, holding a great promise as therapeutic agents. Furthermore, BiP is one of the chaperones responsible for trafficking hIAPP through the ER and Golgi in human β-cells [169]. Similarly, studies with PDI have demonstrated that changes in the catalytic activity of oxidoreductase PDI may contribute to the post-translational defects of hepatic protein secretion in diabetes [170]. Furthermore, experiments conducted *in vitro*, have shown that overexpression of BiP and PDI increased the steady-state intracellular proinsulin levels when exposed to prolonged high glucose [171]. Efforts to understand the impact of chaperones may provide insights into the formation of misfolded hIAPP, which consequently might be a speculative approach for preventing amyloid formation, which may lead to inflammation and β-cell apoptosis in T2D.

During the last decade, there have been some other studies implicating some others ER chaperones in the improvement of T2D. For example, the systemic overexpression of 150-kDa oxygen-regulated protein (ORP150) delayed the onset of disease in heterozygous Akita mice and improved insulin sensitivity [172]. Lastly, recent studies have correlated the role of molecular chaperones with long-term complications of diabetes such as, diabetic peripheral neuropathy (DPN) and diabetic retinopathy. In DPN, Urban et al. reported that modulating molecular chaperones (heat shock proteins 70 and 90) may be effective in improving insulin resistance and indirectly delaying the progression of DNP in T2D [173]. Concerning diabetic retinopathy, the molecular chaperone inducer canavanine might contribute to the improvement in the electrical responses of the diabetic retina [174]. Both studies indicated the potential benefit of molecular chaperones in

decreasing the chronic consequences of diabetes and suggest that enhanced chaperone levels would be an effective strategy for delaying or even preventing T2D.

Chemical chaperones

Another therapeutic purpose that has been extensively investigated relies on chemical chaperones (small molecules with the same function as endogenous proteins without a specific binding site). The use of these agents is based on their non-selective ability in the stabilization of protein conformation, improvement of ER folding capacity and assisting in the trafficking of mutant proteins [175-178]. However, for an effective folding of mutant proteins, the majority of these chemical chaperones may require high concentrations, making them inappropriate for *in vivo* applications. Even so, two chemical chaperones namely 4-phenyl butyric acid (PBA) and taurine-conjugated ursodeoxycholic acid (TUDCA) were approved by US Food and Drug Administration (FDA) for use in humans. For instance, PBA as a low-molecular weight fatty acid has been found to have chaperone-like activities. The use of this agent has been approved for urea-cycle disorder in humans [179-180] and for the treatment of diseases associated with protein misfolding, such as α1-antitrypsin deficiency and cystic fibrosis [181]. On the other hand, TUDCA which belongs to the group of hydrophilic endogenous bile acids has been used as a liver-protecting agent. This chemical chaperone has been shown to have anti-apoptotic properties [182].

Recent reports in animal models, suggest that chemical chaperones can directly activate or deactivate UPR components and can be potentially useful in treating T2D. Ozcan et al. have shown that chemical chaperones PBA and TUDCA reduce ER stress and restore glucose homeostasis in a mouse model of T2D. In this model, the oral chemical chaperone treatment of obese diabetic mice resulted in the normalization of hyperglycemia and restoration of peripheral insulin sensitivity, thus acting as a potential anti-diabetic agent [163]. Another study in pig islets treated with TUDCA have shown an increased in insulin secretion after high glucose stimulation and reduction in ER stress, suggesting a protective role for islets protection in islets isolation for transplantation [183] (**Figure 27**).

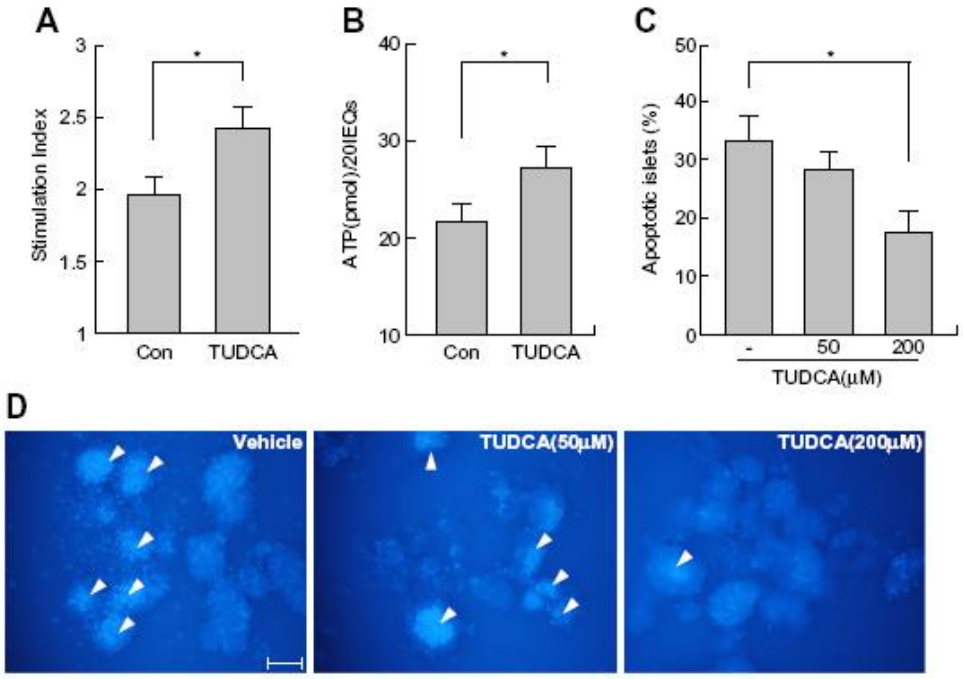


Figure 27. TUDCA increases insulin secretion and viability in islets. TUDCA (200 μM) treatment for 24h immediately after isolation for the analysis of insulin secretion index **(A)** and ATP content of islets **(B)**. In addition, islets were treated with 10 μM of diamidino-2-phenylindole (DAPI) for 15 min and the photos of islets were taken under the fluorescence microscope (20x) **(C)**. Islets undergoing apoptosis were counted and presented as relative percentage (n = 4) **(D)**. Arrow head indicate apoptotic islets. Blue, incorporated DAPI [183].

Chemical chaperones such as PBA have also been tested in obese human subjects showing health benefits by ameliorating insulin resistance and pancreatic β-cell dysfunction in obese subjects [184-185]. These findings offer a new potential approach to improve insulin action and glucose tolerance in diabetic individuals. Further, over the past decade, advances in molecular biology and peptide synthesis have made possible to both identify and synthesize specific peptides capable of inhibiting amyloid aggregation. One of these peptides namely SNNFGA, was shown to attenuate the cytotoxic effects of IAPP on pancreatic β-cell in culture, suggesting that short peptides inhibitors could be considered as therapeutic approaches to treat the toxic effects of islet amyloid deposition in patients with T2D [186].

Nowadays, The ER has been recognizing as an important organelle in deciding cell life and death. Thus, therapeutic interventions that target molecules of the UPR component and reduce ER stress will be promising strategies to treat ER stress-related diseases such as T2D. Moreover, the

ability of endogenous and chemical chaperones to alleviate ER stress in transgenic and obese mice models strongly supports the ER stress-based mechanistic model of T2D and demonstrates the feasibility of targeting ER function for therapeutic goals. The improved understanding of the underlying molecular mechanisms of the UPR in T2D will provide new targets for drug discovery and therapeutic intervention.

5. RATIONALE OF THE THESIS

Understanding the mechanisms regulating whole-body glucose homeostasis is important in order to understand what happens in a disease state such as T2D. One of the major causes involved in the process of β-cell deterioration are the amyloid deposits formed in the pancreas. It was previously described by our group that extracellular hIAPP aggregation is associated with ER stress responses in mouse β-cells. Furthermore, we have recently detected toxic intracellular aggregates in a rat pancreatic β-cell line overexpressing hIAPP, which lead to a defective insulin and IAPP secretion in response to glucose. ER stress and the resulting UPR play a role in β-cell dysfunction. The process of hIAPP misfolding and aggregation is one of the factors that may activate the UPR, perturbing the ER homeostasis. Indeed, many neurodegenerative diseases are associated with accumulation of misfolded proteins in the ER leading to ER stress and progressive cell loss. Similarly, increasing protein synthesis during acute or chronic stimulation and chronic FFA exposure has been reported to induce significant ER stress in β-cells. The combination of glucolipotoxicity and hIAPP overexpression might cause a burden on the ER, not allowing the proteins to reach its native state. On the other hand, molecular chaperones have been described to be important for regulation of ER response to ER stress by stabilizing protein conformation and improving ER capacity. Thus, chaperone ameliorates ER stress induced by hIAPP.

5.1. HYPOTHESIS AND AIMS

Hypothesis

In this dissertation, we attempt to improve our knowledge on hIAPP-induced ER stress and the possible amelioration by chaperone treatment. Our **hypothesis** is that hIAPP potentiates ER stress, and chaperone treatment of pancreatic β-cell models can result in the amelioration of hIAPP-induced ER stress and ultimately diminish amyloid deposits in pancreatic islets. Such findings would provide for new therapies to prevent the loss of β-cell mass associated with amyloid formation in T2D.

Aims

This thesis is aimed to investigate the role of chaperones in β-cell function and amyloid formation under conditions of hIAPP overexpression. In light of this, we have proposed three specific aims:

Aim I. To study the role of endogenously produced hIAPP in response to ER stress induction in a pancreatic β-cell model.

Aim II. To investigate whether endogenous and chemical chaperones are able to recover ER stress and improve insulin secretion in hIAPP cells.

Aim III. To study whether chaperones are able to ameliorate β-cell function and prevent amyloid formation in isolated hIAPP transgenic mouse islets.

Chapter II.
MATERIALS AND METHODS

1. CELL LINE AND PANCREATIC ISLETS

1.1. INS1E CELL LINE

The *in vitro* model used in this study was the rat pancreatic β-cell line INS1E overexpressing human and rat IAPP gene and was prepared as described previously (Soty et al., 2011). Briefly, these cells were stably transfected with hIAPP cDNA (hIAPP-INS1E cells), rat IAPP (rIAPP-INS1E) or an empty vector (INS1E control) under the cytomegalovirus promoter (CMV). After transfection using Lipofectamine, 800 µg/ml Geneticin was added to culture medium for two-weeks. Next, Geneticin-resistance colonies were picked and expanded into stable clones in presence of 200 µg/ml Geneticin.

1.1.1. Maintenance and culture of INS1E cell line

INS1E stable clones were maintained in complete RPMI 1640 (Sigma) supplemented with 1 mM sodium pyruvate (Thermo Scientific), 10 mM HEPES (Thermo Scientific), 10% Fetal Bovine Serum (FBS), 2 mM L-glutamine, 5 µM β-mercaptoethanol, 200 µg/ml geneticin (Gibco®) and 100 units/ml of penicillin and 100 µg/ml streptomycin at 37°C with 5% CO_2. The media was change every 2 days.

1.1.2. Cell splitting

Once the cells reached 70-80% of confluence in 10 cm tissue culture dishes, cells were washed in phosphate buffered saline (PBS; Sigma) before incubation in trypsin/EDTA (Gibco BRL) for 2-3 minutes at 37°C. Cells were resuspended in appropriate media and split for maintenance in 10 cm tissue culture dishes or seeded onto 6 or 12-well-plates, depending on the experiment.

1.2. ANIMAL MODEL: TRANSGENIC hIAPP MOUSE MODEL

For this study we used transgenic human IAPP (Tg hIAPP+/-) mice or non-transgenic littermate controls on FVB background. Animal breeders were obtained from Jackson Laboratories (Bar Harbor, ME, USA). Because islet amyloid occurs more frequently in male than in female hIAPP transgenic mice, only male mice were used in this study. All protocols for animal use and euthanasia were reviewed and approved by the Animal Research Committee of the University of Barcelona.

1.2.1. Islet isolation and culture

10-week-old transgenic mice (wild type or hIAPP Tg) were sacrificed by cervical dislocation and the abdominal cavity was open to allow the exposure of the pancreas and bile duct. After clamping the common bile duct as it joins the intestine, the pancreas was inflated with 1.5 mg/ml of collagenase solution (Roche Diagnostics) diluted in Hanks balanced salt solution (Sigma-Aldrich). The distended pancreas was removed and incubated at 37°C for 6 minutes 30 seconds, and the islets were dispersed by gentle shaking. Enzymatic digestion was stopped by the addition of cold Hanks-BSA solution. After centrifugation (100 × g for 5 minutes at 4°C) islets were resuspended in 10 ml of Hanks-BSA. The slurry was layered on top of a prepared Histopaque gradient (comprised of a 10 ml lower layer of Histopaque 1119, and 10 ml upper layer of Histopaque 1077 (Sigma-Aldrich). Following centrifugation of the gradient at 1000 × g for 20 minutes at room temperature (RT), islets were collected from the top of the 1077 interface, pipetted into a 50 ml tube (Falcon) and refilled with cold Hanks-BSA to 50 ml and centrifuged at 500 x g for 5 minutes at 4°C. Finally, the supernatant was discarded and the pellet resuspended in Hanks-BSA solution. Then, islets were rinsed into a Petri dish, isolated from any contaminating exocrine material visually using a dissecting microscope, and cultured in RPMI media containing 10% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin and at 37°C in a 5% CO_2 incubator to recover before performing the experiments. Prior to all experiments, islets were cultured for 24 hours in RPMI media containing 11 mM glucose.

2. PALMITATE PREPARATION

Palmitate (Sigma) was solubilized in 10 ml of Absolute Ethanol to make a 50 mM of stock solution. The solution was filtered, sterilized and stored in aliquots at -20°C until required.

3. TREATMENT OF INS1E CELLS AND MOUSE ISLETS

Palmitate (400 µM) combined with 25 mM glucose was added to the cells and islets by conjugating with 1% (w/v) of Albumin from Bovine Serum (BSA, Sigma) dissolved in 10% FBS RPMI-1640. Thapsigargin (Sigma) was diluted in DMSO and used at a final concentration of 0.5 µM. Sodium phenylbutyrate (4-PBA) (Sigma) was dissolved in PBS and used at 2.5 mM. Taurousodeoxycholic Acid (TUDCA) (Calbiochem) was diluted in RNAse-free water and used at 200 µM. All reagents were added to the cells and islets in fresh medium for 8 and/or 24 hours for gene and protein expression.

4. INS1E CELLS AND MOUSE ISLETS RNA EXTRACTION

At the end of treatment of INS1E clones, the media was discarded and 500 µl of TRIZOL reagent (Invitrogen) was added in the well. The plates were put in agitation for 2-3 minutes and TRIZOL recovered in tubes. In the case of RNA extraction from islets, the tubes containing islets were centrifuged at 1000 rpm for 1 minute and the supernatant discarded. The pellet was resuspended in 500 µl PBS and centrifuged again at 1000 rpm for 1 minute. The resulting supernatant was discarded and the pellet resuspended in 1 ml of TRIZOL reagent. Total RNA from INS1E cells and pancreatic islets was extracted following the manufacturer's instructions and eluted in RNAse-free water. The samples were stored at -20°C until required. The concentration of RNA in the different samples was determined by measurement of the absorbance at 260 nm using a Nanodrop 1000 spectrophotometer (TermoFisher Scientific, Waltham, Massachusetts, USA).

5. cDNA SYNTHESIS

To prepare cDNA from RNA, 0.5-1 µg of total RNA from INS1E cells and 0.25-0.5 µg of total RNA from islets was brought up in a total volume of 10 µl with RNAse-free water. cDNA synthesis was extracted following the Superscript Reverse Transcriptase III instructions (Invitrogen). Briefly, RNA was complexed with 1 µL of dNTPs (10 Mm) (GeneCraft), 1 µL Random Primers and 1 µL of RNAse-free water. The solution was heated at 65°C for 5 minutes and placed back on ice for at least 1 minute. Next, 4 µL of first strand buffer (5x) (Invitrogen), 1 µL of DDT (0.1 M), 1 µL of RNAse Out (40U/µl) (Thermo Scientific) and 1 µL of Superscript Reverse Transcriptase III (200U/µl) was then added followed by gentle mixing and a quick centrifugation and placed in ice.

6. QUANTITATIVE PCR ANALYSIS

Real-Time PCR (RT-PCR) was carried in duplicates with 2 µg of transcribed cDNA and MESA Green qPCR MasterMix Plus FOR SYBR (Eurogentec) in a LightCycler® 480 II sequence detection system (Roche Applied Science). The final volume reaction was 10 µl, and the primers were always used at a final concentration of 10 µM. Briefly, cDNA (4 µl/well) was added to a qPCR mixture (10 µl/well). PCR products were verified using dissociation curve analysis using SDS software (Roche Applied Science). Expression levels were normalized to TATA box-binding protein (Tbp) mRNA and represented in arbitrary units. Primer sequences are provided below in **Table 2**.

Table 2. List of primer sequences used in qRT-PCR experiments.

Primer	(5'----3')	Species	Gene ID
ATF3 Forward	GCTGGAGTCAGTCACCATCA	Rat	Atf3
ATF3 Reverse	ACACTTGGCAGCAGCAA	Rat	Atf3
CHOP Forward	CCAGCAGAGGTCACAAGCAC	Rat	Ddit3
CHOP Reverse	CGCACTGACCACTCTGTTTC	Rat	Ddit3
Spliced XBP1 Forward	GAGTCCGCAGCAGGTG	Rat	Xbp1
Spliced XBP1 Reverse	GCGTCAGAATCCATCCATGGGA	Rat	Xbp1
TBP1 Forward	GAGATCACCCTGCAGCATCA	Rat	Tbp
TBP1 Reverse	GCAGTGCCGCCCAAGTAG	Rat	Tbp
BiP Forward	TGCAGCAGGACATCAAGTTC	Rat	Hspa5
BiP Reverse	AAAGAAGACCCCGTTTACAG	Rat	Hspa5
ATF3 Forward	TCGGATGTCCTCTGCGCTGGA	Mouse	Atf3
ATF3 Reverse	CTGACTCTTTCTGCAGGCACTCTGT	Mouse	Atf3
CHOP Forward	AAGATGAGCGGGTGGCAGCG	Mouse	Ddit3
CHOP Reverse	GCACGTGGACCAGGTTCTGCT	Mouse	Ddit3
Spliced XBP1 Forward	GAACCAGGAGTTAAGAACACG	Mouse	Xbp1
Spliced XBP1 Reverse	AGGCAACAGTGTCAGAGTCC	Mouse	Xbp1

7. DETERMINATION OF CELL VIABILITY

7.1. LIVE/DEAD® ASSAY

LIVE/DEAD® Viability/Cytotoxicity Kit (Invitrogen) was performed following manufacturer instructions. Briefly, adenoviral transduced cells were cultured in the presence of 20 MOI of Ad-BiP. Cells were washed with PBS and combined LIVE/DEAD assay reagents were applied for 30-45 minutes at RT. Thapsigargin treatment for 24 hours was used as positive control. Images were captured using an Olympus Bx61 microscope and In Vivo or DP controller software.

8. BiP, PDI AND GFP ADENOVIRAL TRANSDUCTION AND CELL INFECTION

The recombinant adenoviruses encoding for BiP under the CMV promoter were kindly provided by Dr. V. Petegnief (IDIBAPS, Barcelona). Ad-CMV-PDI/GFP was kindly provided by Dr. A. Volchuk (University Health Network of Toronto, Canada). Ad-GFP was kindly provided by Dr. R. Gasa (IDIBAPS, Barcelona).

INS1E cells ($5x10^5$/12-well plate) and isolated pancreatic islets (8-10 per condition) were transduced with 20 MOI of Ad/CMV-BiP, Ad/CMV-PDI or Ad/CMV-GFP and incubated at 37°C and 5% CO_2 for 2 hours. INS1E cells or mouse islets were then washed with PBS and incubated in a transfection medium containing no antibiotics and 1% FBS at 37°C and 5% CO_2 for 24 hours in complete RPMI prior to further treatment. After the treatments, the cells were washed once again with PBS and lysed for either GSIS or Western blot analysis as described below.

9. SMALL INTERFERING RNA TRANSFECTION (siRNA)

Approximately $3x10^5$ hIAPP-INS1E cells were seeded in a 12-well-plate for 24 hours. Knockdown of CHOP expression in cells was performed using 20 µM of small interfering RNA (Invitrogen) or 20 nM of scramble siRNA as a control using METAFECTEN®PRO (Biontex). Briefly, per transfection, 20 nM of siRNA (Invitrogen) or 20 nM of scramble siRNA was diluted in 60 µl of

transfection reagent medium solution without serum and incubated at RT for 20 minutes. After incubation, the lipid-complexes solution was added by dropwise to the cells and kept at 37°C in a CO_2 incubator. Six to eight hours later, the lipid solution was changed to complete RPMI. Twenty-four hours after transfection, cells were treated with either thapsigargin or HG (25 mM) and BSA-coupled PA (400 µM) for another 24 hours.

10. GLUCOSE-STIMULATED INSULIN SECRETION (GSIS) ASSAY

hIAPP-INS1E cells were seeded in 12-well plates ($5x10^5$) at 11 mM of glucose. After 2 days cells were transduced overnight with 20 MOI of Ad-GFP, Ad-BiP, Ad-PDI or treated with 200 µM TUDCA or 2.5 mM PBA. After 24 hours of transduction or chemical chaperone treatment, cells were cultured at high glucose and palmitate (HG+PA) or kept at basal conditions (11 mM) for another 24 hours. Freshly isolated wild type and hIAPP transgenic mouse islets were transduced overnight with 20 MOI of Ad-BiP, Ad-PDI, Ad-GFP or treated with TUDCA and PBA.

INS1E cells and islets were preincubated in triplicate with Krebs-Ringer bicarbonate buffer (KRB) containing 140 mM NaCl, 4.5 mM KCl, 2.5 mM $CaCl_2$, 1 mM $MgCl_2.6H_2O$, 20 mM HEPES, pH 7.4 and supplemented with 0.1% BSA and 2.8 mM glucose for 30 minutes at 37°C with 5% CO_2, following by stimulation with 2.8 mM or 16.7 mM glucose KRB for 1 hour at 37°C. At the end of the experiment, the supernatant was recovered and INS1E/islets were lysed in 500 µl of acid-ethanol solution to measure insulin content. Insulin levels and contents were determined by insulin ELISA (Mercodia) according to manufacturer`s protocol.

11. IMMUNOHISTOCHEMISTRY

11.1. INS1E CELL LINE

hIAPP-INS1E cells were seeded in 24-well plates (~$10x10^4$). After an overnight incubation cells were treated as indicated above. Following treatments, cells were washed twice with PBS and fixed in 4% paraformaldehyde for 10 minutes at RT. After two washes in PBS cells were blocked

with PBS in 0.2% FBS for 1 hour at RT, following the addition of guinea pig anti-insulin (Dako, 1:100) primary antibody and rabbit anti-caspase 3 antibodies (Cell Signaling, 1:100) and goat anti-rabbit Alexa Fluor 594 (Molecular Probes, 1:100) as secondary antibody.

11.2. MOUSE ISLETS

Isolated islets were fixed in 4% paraformaldehyde for 45 minutes. After two washes in PBS, islets were placed in 55°C agarose (Sigma) and spun down at 1000 rpm for 20 seconds. Agarose-embedded islets were dehydrated using a set of graded ethanol solutions: 50% ethanol, 70% ethanol, 95% ethanol, 100% ethanol all of them were left for 30 minutes at RT and at the end in xilol for 1 hour at RT. After removing xilol solution islets were embedded in paraffin. The sections were cut using a microtome and mounted, and then hydrated in series of graded ethanol solutions:

1) Xylene 2x for 10 minutes each,

2) 100% ethanol 2x times for 10 minutes each,

3) 95% ethanol for 5 minutes,

4) 70% ethanol for 5 minutes,

5) 50% ethanol for 5 minutes. Then the slides were rinsed with deionized water. Sections were immunostained for insulin as described above. Amyloid staining was performed by ThioS staining. Briefly, slides were leave for 2 minutes in ThioS bath. Next, slides were dipped for two times in 70% ethanol for 15-20 minutes each. Slides were then dipped in deionized water for 5 minutes. Finally, a drop of mounting solution was added before being cover slipped.

12. PROTEIN TECHNIQUES

12.1. PROTEIN EXTRACTION FROM INS1E CELLS AND ISLETS

INS1E cells were scraped off 10 cm diameter culture plates in 100 µl solution of ice cold lysis buffer and 10% of proteases inhibitor Cocktail (Sigma) and transferred to a clean microfuge tube and put on ice for 20 minutes. The whole cell lysate was then centrifuged at 12'000 x g for 15 minutes at 4°C. The supernatant was removed and transferred to a new microfuge tube.

In the case of islets, following experimentation islets were recovered in clean eppendorfs and centrifuged at 1000 x g for 1 minute and the supernatant discarded. Lysis buffer supplemented with 10% of protease inhibitor cocktail (Sigma) was added and vortex for 30 seconds and put on ice for 20 minutes. The whole cell lysate was then centrifuged at 12'000 x g for 15 minutes at 4°C. The supernatant was removed and transferred to a new microfuge tube. Protein concentration in lysates was determined by Bio-Rad Protein Assay kits (Bio-Rad), following manufacturer's instructions. Samples were stored at -20°C until required.

12.2. WESTERN BLOTTING

Protein lysate samples (20-30 µg) were resolved by 8-10% SDS-PAGE (sodium dodecyl sulfate polyacrilamide gel electrophoresis) gels and transferred to 0.45-µm PVDF membranes (Perkin Elmer Life Sciences). Membranes were previously soaked in 100% methanol to wet them. The gel was then blotted for 50 minutes at 100 mV. Membranes were blocked for 2 hours with 5% skim milk or 5% BSA in wash buffer TBST and incubated overnight at 4°C in primaries antibodies (**Table 3**). Membranes were washed 3x with TBST and incubated with horseradish Peroxidase-conjugated secondary antibodies (GE Healthcare) for 1 hour. Immunoreactive protein bands were developed with ECL chemiluminescence reagents kit (Pierce) following manufacturer´s instructions.

Table 3. List of primary and secondary antibodies.

Target	Manufactured	1º antibody source	Dilution (TbsT)	Molecular weight (kDa)	2º Antibody	Dilution (TbsT)
BiP	Santa Cruz (C-20):1051	Goat	1:1000 5% milk	78	Anti-goat IgG, peroxidase A5420	1:5000 5% milk
p-eIF2α	Cell Signalling Ser51	Rabbit	1:1000 5% BSA	38	Anti-rabbit IgG, peroxidase NA934	1:5000 5% BSA
ATF3	Santa Cruz (C-19):188	Rabbit	1:1000 5% milk	21	Anti-rabbit IgG, peroxidase NA934	1:5000 5% milk
CHOP	Cell Signalling L63F7	Mouse	1:1000 5% BSA	28	Anti-mouse IgG, peroxidase NA931	1:5000 5% BSA
β-Actin	Sigma A2066	Rabbit	1:1000 5% milk	42	Anti-rabbit IgG, peroxidase NA934	1:5000 5% milk
Tubulin	Sigma	Sigma T6074	1:1000	50	Anti-mouse IgG, peroxidase NA931	1:5000 5% milk

12.3. BUFFERS AND REAGENTS

Lysis Buffer 1 ml Triton X-100% 10 mM sodium phosphate 10 mM sodium fluorite 50 mM Tris-HCl pH 7.5 5 mM EDTA 150 mM NaCl	**Laemmli Sample Buffer (2X)** 0.5 M Tris pH 6.8 10% w/v SDS 40% v/v Glycerol 10% v/v b-mercaptoethanol 20 mg/ml Bromophenol blue
SDS-PAGE Running Buffer (10x) 25 mM Tris 192 mM Glycine 0.1% w/v SDS	**TBS-Tween (TBST-0.05%)** Destilated water Tween-20 TBS 20x
Transfer Buffer (1x) Glycine Tris Methanol	

13. STATISTICAL ANALYSIS

Statistical analysis between two groups was performed using Student's two-tailed t test and differences among more than two groups were carried out by ANOVA followed by Tukey test. Differences were considered significant when $*p < 0.05$. Data in bar graphs are represented as mean ± S.E.M.

Chapter III.
RESULTS

AIM I

To study the role of endogenously produced hIAPP in response to ER stress induction in a pancreatic β-cell model

1. INDUCTION OF ER STRESS AND APOPTOSIS IN hIAPP-INS1E CELLS

Thapsigargin is known to induce ER stress by causing a depletion of calcium levels within the lumen of the ER. Here we examine the effect of thapsigargin on ER stress response in our cell models. INS1E cells were cultured at basal conditions and treated with 1 µM of thapsigargin for 8 and 24 hours. As expected, after treatment with thapsigargin, INS1E cells showed increased expression levels of ER stress genes such as *activating transcription factor 3* (ATF3), *spliced X-box binding protein 1* (sXBP1) and *CCAAT/enhancer-binding protein (C/EBP) homologous protein* (CHOP) at 8 hours. The increase was maintained or increased at 24 hours (**Figure 28**).

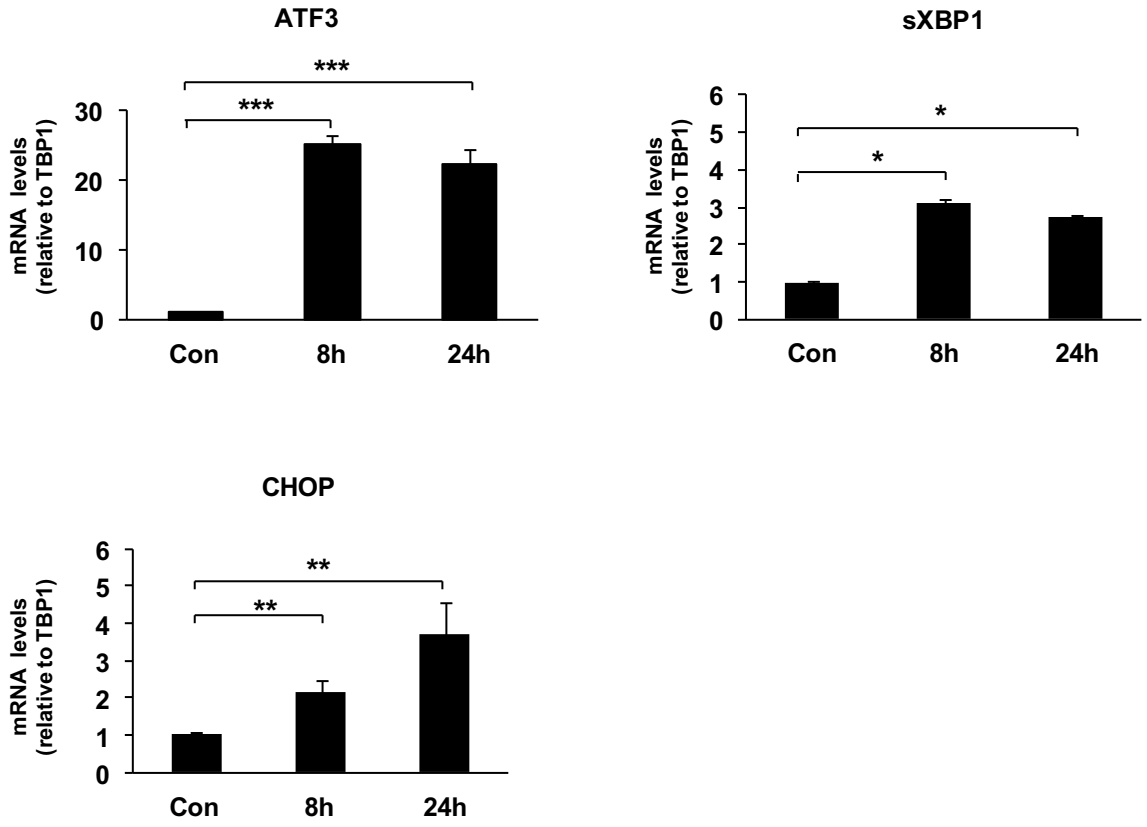


Figure 28. Thapsigargin treatment increases ER stress markers in a time-dependent manner in INS1E cells. INS1E control cells were cultured at 11 mM glucose and treated with 1 µM of thapsigargin for 8 and 24 hours. *mRNA* levels of ER stress markers *ATF3*, *sXBP1* and *CHOP* were quantified by Real-Time PCR. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ *vs* Con cells at time 0. Results are expressed as mean ± S.E.M from five independent experiments.

Previous studies have shown that hIAPP induces apoptosis and ER stress by increasing the levels of ER stress markers [2-3]. In line with these findings we examined whether hIAPP overexpression potentiates ER stress in β-cells. In our lab, we created an INSE1 β-cell line model of hIAPP overexpression, where INS1E cells stably express human IAPP (hIAPP-INS1E) or rat IAPP (rIAPP-INS1E) as control. Thus, we treated hIAPP-INS1E cells, rIAPP-INS1E cells and INS1E cells with thapsigargin and determined the effect on ER stress gene expression. Although thapsigargin was able to induce gene expression on key ER stress genes, as shown in **Figure 28**, treatment with 1 μM thapsigargin for 24 hours did not have any effect in protein levels of CHOP or cleaved caspase 3 and did not affect the expression levels of insulin or IAPP in INS1E control cells (**Figure 29, left panel**). However, in hIAPP-INS1E cells, treatment with 1 μM of thapsigargin for 24 hours induced ER stress and severe apoptosis, as seen by activation of CHOP and cleaved caspase 3 (**Figure 29, right panel**). In addition, thapsigargin treatment also resulted in decreased insulin and hIAPP protein levels (**Figure 29, right panel**), indicating that the dose corresponding to 1 μM of thapsigargin was associated with a high toxicity in hIAPP-INS1E cells.

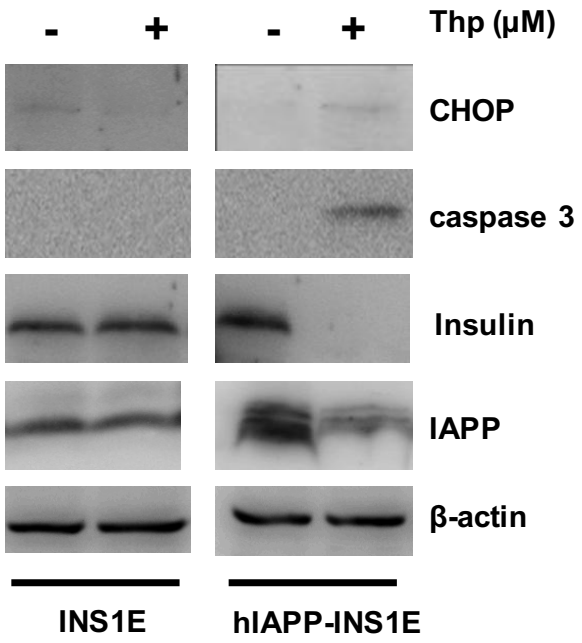


Figure 29. Thapsigargin induces severe ER stress and apoptosis in hIAPP-INS1E cells. INS1E and hIAPP cells were exposed to 1 μM thapsigargin (Thp) for 24 hours. Expression levels of CHOP, cleaved caspase 3, insulin, IAPP and β-actin were determined by Western blot.

Since 1 µM thapsigargin was associated with severe cell death and toxicity we next aimed to determine a lower dose in which cells get a milder activation of ER stress. Thus, we performed a dose/response experiment (0.25, 0.5 and 1 µM) at 8 and 24 hours. hIAPP-INS1E cells showed a high activation of ER stress marker CHOP and effector cleaved caspase 3 at 24 hours (**Figure 30**). Furthermore, hIAPP-INS1E cells lost hIAPP expression, suggesting that 0.5 or 1 µM thapsigargin at 24 hours still proved to be lethal in these cells (**Figure 30**). Nevertheless, the dose of 0.5 µM of thapsigargin at 8 hours showed a mild activation of CHOP, absence of cleaved caspase 3 and unaffected hIAPP expression. Thus, a dose of 0.5 µM for 8 hours was chosen for further experiments based on optimal ER stress induction in the absence of cell dysfunction or apoptosis.

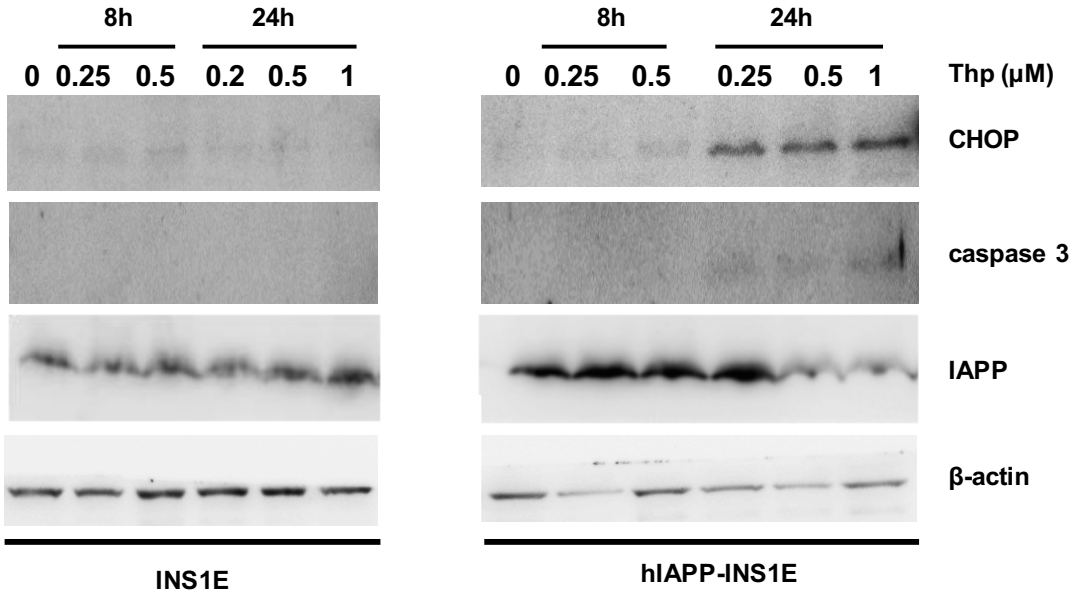


Figure 30. Thapsigargin time- and dose-dependent treatment. INS1E and hIAPP-INS1E control cells were exposed to 0.25, 0.5 and 1 µM of thapsigargin (Thp) for 8 and 24 hours. Protein levels of CHOP, cleaved caspase 3, IAPP and β-actin were determined by Western blot (n=3). Representative Western blotting images are shown from to 3 independent experiments.

2. hIAPP-INS1E CELLS ARE MORE SENSITIVE TO ER STRESS INDUCERS

In order to study whether hIAPP overexpression was associated with alterations in levels of ER stress markers we examined the expression of ER stress markers in hIAPP-INS1E cells when compared to controls (rIAPP and INS1E cells) at basal conditions. As expected**,** hIAPP-INS1E cells cultured at 11 mM of glucose did not show changes in ER stress genes, such as *CHOP*, *sXBP1* or *ATF3*, when compared to rIAPP-INS1E or INS1E control cells (**Figure 31A**). We have previously shown that a dose of 0.5 µM of thapsigargin was associated with a mild increase in ER stress. Thus, when cells were exposed to 0.5 µM thapsigargin for 8 hours, the expression of *CHOP* and *ATF3* was significantly higher in hIAPP-INS1E when compared to rIAPP-INS1E or INS1E control cells (**Figure 31B**), suggesting that hIAPP-INS1E cells were more sensitive to chemical ER stress inducers.

High levels of glucose and fatty acid treatment has been shown to activate ER stress pathways in β-cells. Thus, we next investigated the effect of physiological ER stress inducers such as high glucose (HG) and palmitate (PA) on ER stress genes in hIAPP-INS1E cells. After 8 hours culture of hIAPP-INS1E, rIAPP-INS1E and INS1E cells treated with 25 mM glucose or 400 µM PA separately, cells did not show changes in gene expression as compared to basal 11 mM glucose (**Figure 31 C,D**). Conversely, hIAPP-INS1E cells treated with HG together with PA, significantly increased *mRNA* levels of ER stress markers such as *CHOP*, *ATF3* and *sXBP1* when compared to rIAPP and INS1E cells (**Figure 31C**). These results indicate that treatment with HG and PA, together with the overexpression of hIAPP senses the ER and triggers the activation of the UPR pathway, making hIAPP-INS1E cells more sensitive to ER stress than rIAPP-INS1E or INS1E controls.

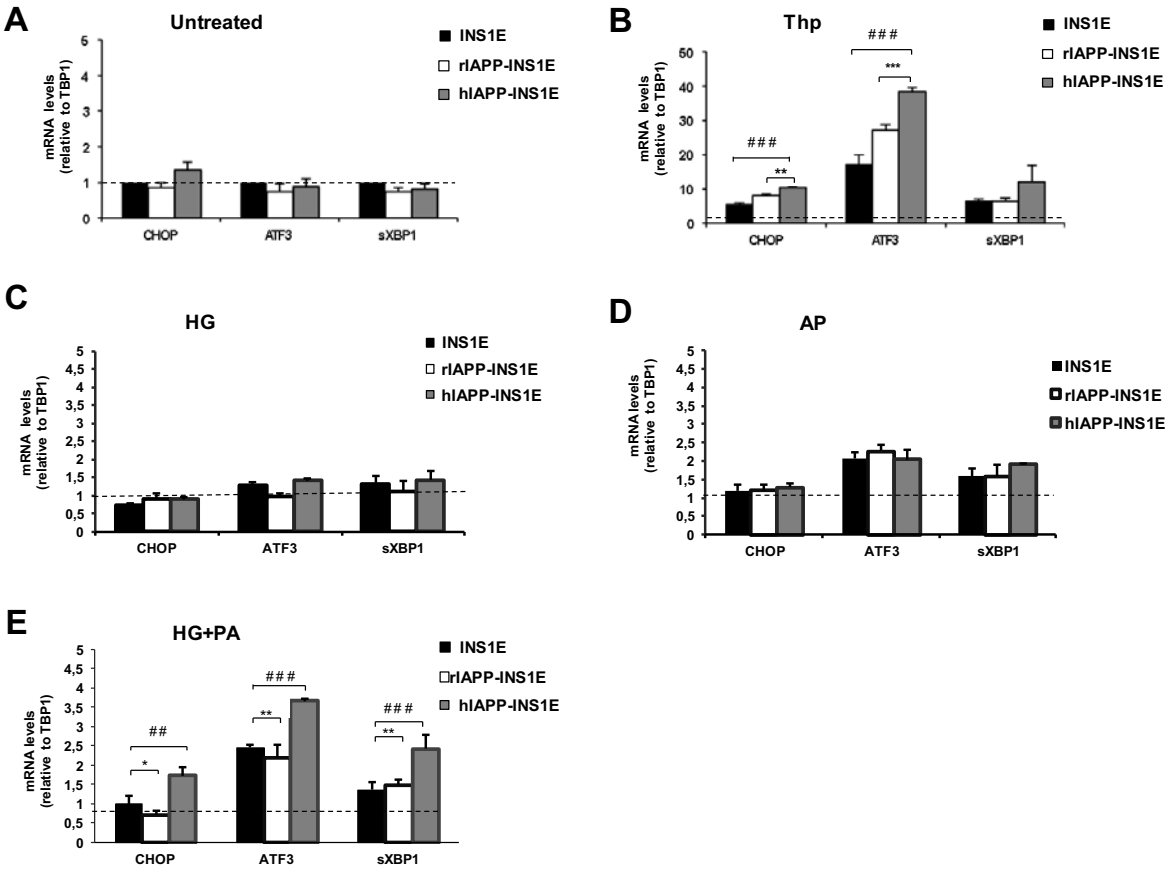


Figure 31. Thapsigargin and high glucose and palmitate treatments potentiate ER stress gene expression in hIAPP-INS1E cells. ER stress expression markers *CHOP*, *ATF3* and *sXBP1* were determined by Real-Time PCR from hIAPP-INS1E, rIAPP-INS1E and INS1E control cells cultured at **A)** 11 mM glucose (untreated) **B)** 11 mM glucose exposed to 0.5 µM of thapsigargin (Thp) for 8 hours or **C)** 25 mM glucose (HG) **D)** 400 µM Palmitate (PA) and **E)** HG+PA for 24 hours. Results are normalized to untreated INS1E, rIAPP-INS1E or hIAPP-INS1E cells (dashed line) and expressed as mean ± S.E.M from five independent experiments. $*p < 0.05$ $**p < 0.01$ and $***p < 0.001$ *vs* rIAPP and $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ *vs* INS1E control.

3. CHOP INHIBITION PROTECTS FROM INDUCED ER STRESS AND APOPTOSIS IN hIAPP-EXPRESSING INS1E CELLS

We next tested whether CHOP is a key component in the ER stress-apoptosis pathway in our cell model of hIAPP overexpression under stressful conditions. Thus, interfering RNA siCHOP was used to knockdown the expression of CHOP and a scrambled siRNA was used as a control. hIAPP-INS1E cells were transfected for 24 hours with 20 nM of both siRNAs. Twenty-four hours after transfection cells were treated for 8 hours with 0.5 µM thapsigargin or HG (25 mM) and PA (400

µM). As expected, treatment with thapsigargin or HG+PA induced the expression of CHOP. In contrast, the protein levels of the upstream ER stress marker ATF3 were not affected (**Figure 32 A,B**). Moreover, when hIAPP-expressing cells were transfected with siCHOP, thapsigargin or HG+PA treatment failed to induce CHOP expression (**Figure 32 A,B**) confirming successful knockdown of CHOP, whereas scrambled siRNA showed similar levels of CHOP when compared to treated controls. These results suggest that ER stress induction by thapsigargin and HG and PA is dependent on CHOP activation.

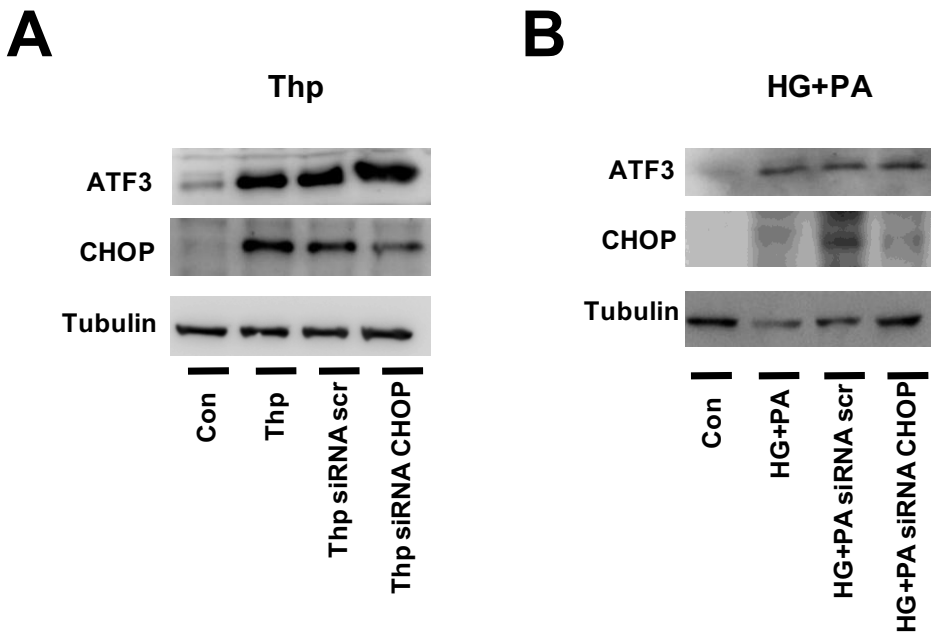


Figure 32. Knockdown of CHOP prevents ER stress induction in hIAPP-INS1E cells. hIAPP-INS1E cells were transfected with 20 nM of siRNA CHOP or siRNA scramble (siRNA scr) as a control. Twenty-four hours after transfection, cells were treated with **A)** 0.5 µM Thp for 8 hours or **B)** HG (25 mM) and BSA-coupled PA (400 µM; HG+PA) for 24 hours, and protein expression levels for CHOP, ATF3 and Tubulin were determined. Representative Western blotting images are represented from 3 to 5 individual experiments.

We next examined whether decreased in CHOP expression was associated with a reduction in downstream of the apoptotic marker cleaved caspase 3. Thapsigargin or HG+PA treatment induced activation of caspase 3 when cells were treated with siRNA scramble as compared with control. In contrast, caspase 3 was completely blunted with deletion of CHOP (**Figure 33 A,B**),

suggesting that the effects observed are CHOP mediated, since the knockdown of CHOP alone is sufficient enough to prevent induced thapsigargin and HG+PA apoptosis.

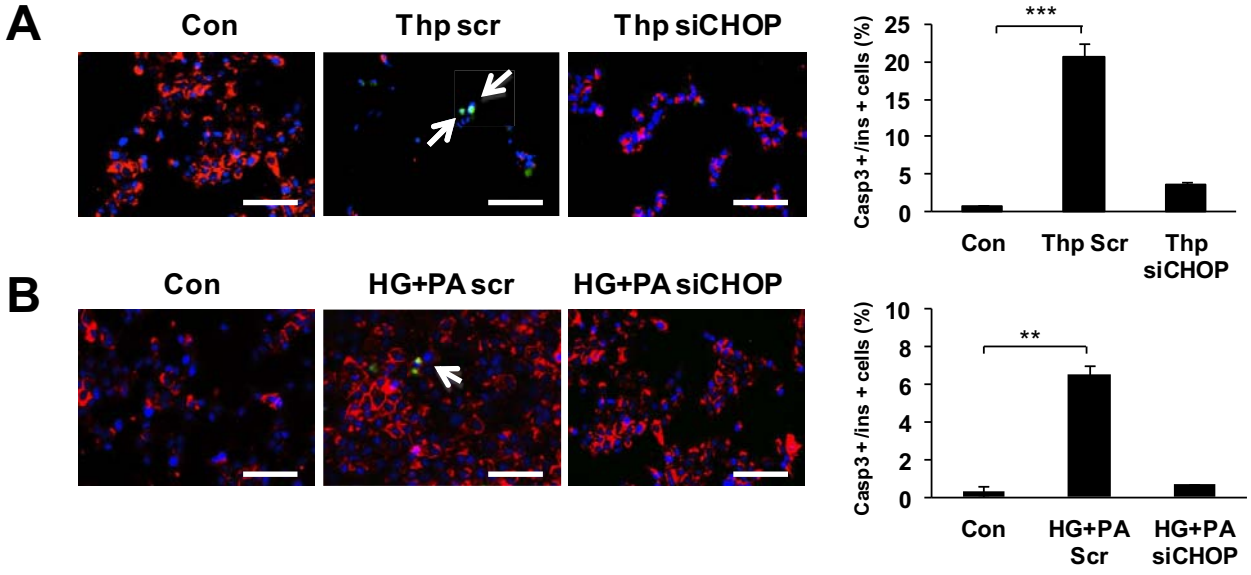


Figure 33. Knockdown of CHOP protects hIAPP-INS1E cells from induced apoptosis. Immunostaining (left panel) and quantification (right panel) of insulin positive β-cells (red), cleaved caspase 3 (green) and nuclei (blue) of hIAPP-INS1E cells (Con) and hIAPP-INS1E cells transfected with siRNA scr or siRNA CHOP previously treated with **A)** Thp or **B)** HG+PA. Note the absence of cells containing both insulin and cleaved caspase 3 staining in cells transfected with siRNA CHOP as compared to Thp or HG+PA treated controls. Scale bar is 50 µm. Quantification is normalized to number of insulin + cells and expressed as mean ± S.E.M from three independent experiments. $^{**}p < 0.01$ and $^{***}p < 0.001$ *vs* controls. No statistical differences were found between controls and Thp siCHOP or HG+PA siCHOP.

AIM II

To investigate whether endogenous and chemical chaperones are able to recover ER stress and improve insulin secretion in hIAPP cells

4. CHAPERONES AMELIORATE INDUCED ER STRESS IN hIAPP-INS1E CELLS

Molecular and chemical chaperones administration has been used as a strategy to diminished ER stress among animal models of T2D. According with this, we hypothesized that overexpression of molecular chaperones in β-cells could ameliorate β-cell stress and function in our cell model of hIAPP overexpression. Therefore, to increase endogenous chaperone expression, we used adenoviral vectors encoding for chaperones BiP and PDI. Recombinant adenoviruses are commonly used to achieve more robust transduction efficiency as pancreatic β-cells are difficult to transduce with other conventional methods. First, we amplified and purified an adenovirus encoding for the chaperone BiP (Ad-BiP). Western blot analysis after Ad-BiP transduction showed increased BiP protein expression with increasing doses (multiplicity of infection or MOI) of adenovirus (at 2, 5, 20 and 40 MOI) in hIAPP-INS1E for 24 hours (**Figure 34**).

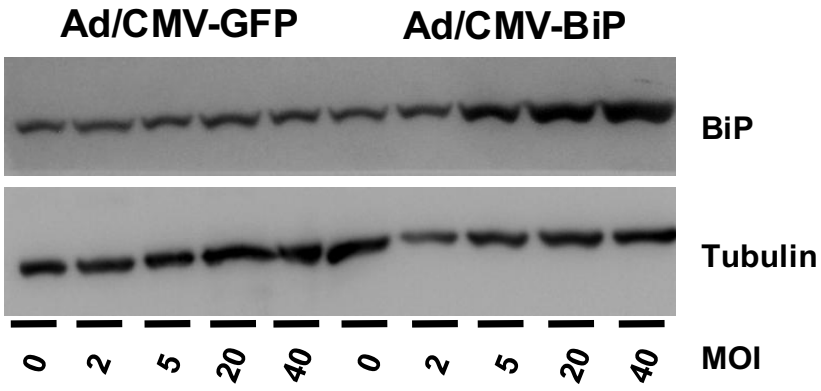


Figure 34. Increased BiP expression after adenoviral transduction at different MOI. hIAPP-INS1E cells were transduced with different doses (MOI) for 24 hours. Representative Western blotting shows BiP protein levels at indicated doses. Western blotting is representative from 3 independent experiments.

Similarly, we amplified and purified an adenoviral vector coding for PDI coupled to the green fluorescence protein (Ad-PDI/GFP). Ad-PDI/GFP was similarly tested in hIAPP-INS1E cells by transducing with Ad-PDI/GFP for 24 hours at different MOIs. The PDI/GFP expression was determined by fluorescence microscopy, showing a gradually increase of GFP at increasing MOI (**Figure 35A**).

In order to supervise the viability of cells after transduction with adenovirus we performed a cytotoxicity assay, which discriminates live from dead cells due to a green-fluorescent protein that indicates intracellular esterase activity (alive cells) and red-fluorescent protein indicating the loss of plasma membrane integrity (death cells). As a positive control of cell death, non-transduced cells were treated with 5 µM of thapsigargin. Cell death was observed after 24 hours of treatment with thapsigargin. At the same time, cells transduced with 20 MOI of Ad-BiP did not show any indicator of cell death (**Figure 35B**). Therefore, a MOI of 20 for each adenovirus was chosen for future experiments, based on maximal BiP or PDI/GFP expression in the absence of cell death or detectable cell toxicity.

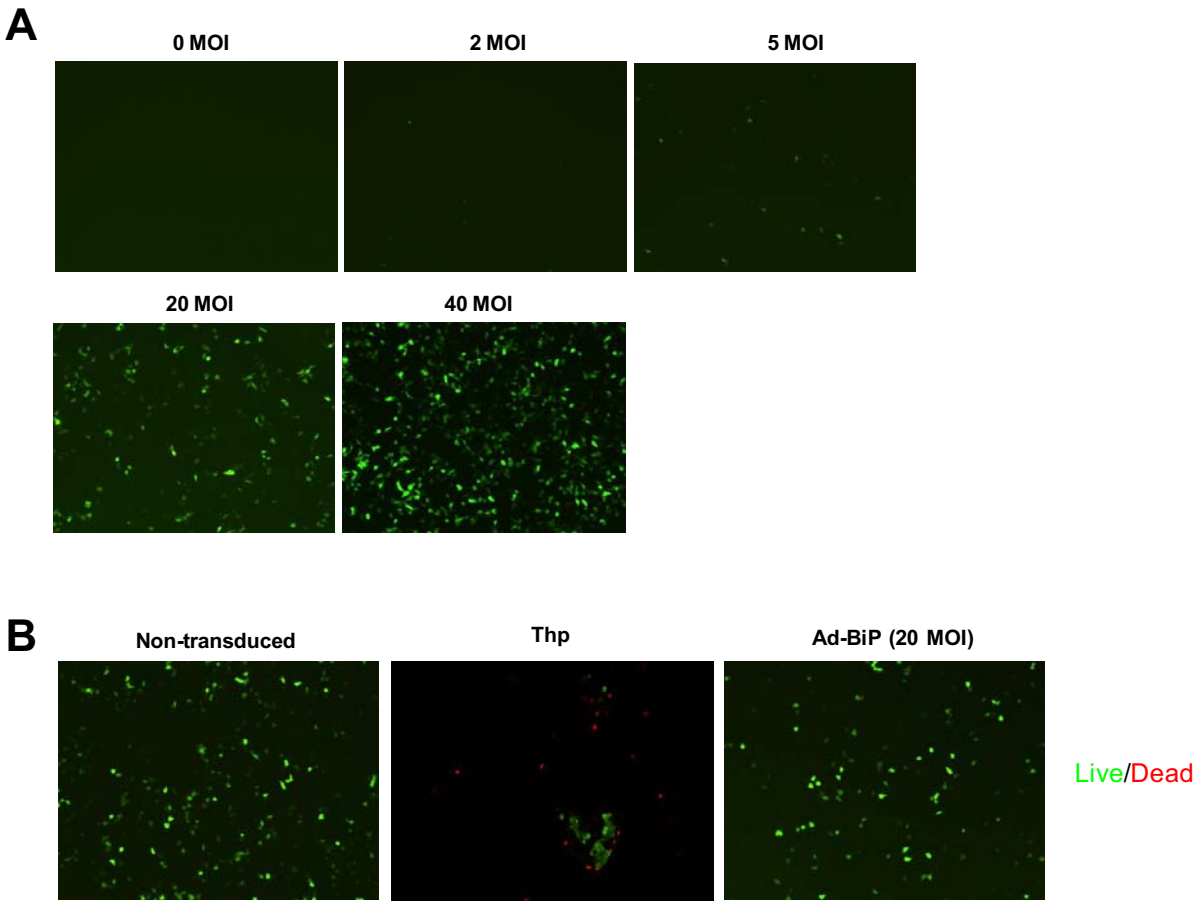


Figure 35. Increased BiP and PDI expression after adenoviral transduction does not affect cell viability. A) GFP expression after adenoviral (Ad-PDI/GFP) transduction in hIAPP-INS1E cells after 24 hours. Note an increase in GFP expression that correlates with an increase in MOI. **B)** Live/death viability assay showing no cell death in hIAPP-INS1E cells transduced for 24 hours with Ad-BiP.

Once transduction conditions were set up and with the purpose to investigate the role of chaperones in ER stress under physiological and non-physiological stress conditions, hIAPP-INS1E cells were transduced with the molecular chaperone BiP or treated with chemical chaperones TUDCA and PBA and cells were exposed to HG and PA conditions.

We first tested whether BiP could be overexpressed under stressful conditions. hIAPP and rIAPP cells were transduced with 20 MOI of Ad-BiP and Ad-GFP for 24 hours. Then, hIAPP-transduced cells were treated with thapsigargin or HG+PA for 8 hours. As shown in **Figure 34,** BiP was highly expressed under basal conditions and after treatment with HG and PA (**Figure 36A**). Surprisingly, BiP was not overexpressed in either hIAPP and rIAPP cells in the presence of thapsigargin (**Figure 36A**), suggesting that thapsigargin blunted adenoviral protein expression. Next, hIAPP-INS1E cells were cultured in the presence of thapsigargin-containing chaperones TUDCA and PBA for 24 hours. After exposure to thapsigargin, hIAPP-INS1E cells showed high protein expression levels of ER stress markers CHOP and ATF3 (**Figure 36B**), confirming the mRNA expression results observed in **Figure 28**. Interestingly, when hIAPP-INS1E cells were treated with chaperones TUDCA and PBA, CHOP and ATF3 levels were significantly diminished (**Figure 36B**), although BiP levels were not recovered. Together, these results suggest that chemical chaperones TUDCA and PBA were able to reduce the hIAPP-potentiated ER stress associated with thapsigargin.

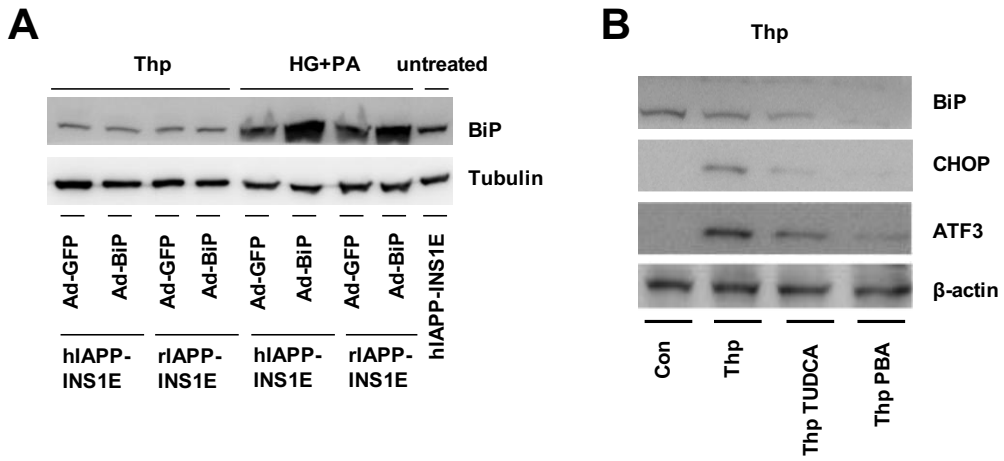


Figure 36. BiP overexpression and decrease of ER stress markers by chemical chaperones in hIAPP-INS1E cells. A) BiP and tubulin levels were determined in hIAPP-INS1E cells previously transduced with Ad-BiP or Ad-GFP for 24 hours. Representative western blotting results showing protein expression levels of CHOP, ATF3, BiP, and β-actin levels in hIAPP-INS1E cells cultured with **B)** 0.5 μM Thp for 8 hours. Representative results from 3 to 5 individual experiments are shown.

To mimic the plausible protective role of chaperones during HG+PA-induced ER stress, hIAPP-INS1E cells were incubated with HG and PA and transduced with Ad-BiP or Ad-PDI for 24 hours or treated with TUDCA and PBA. As expected, hIAPP-INS1E cells exposed to HG+PA showed an increase in ER stress genes such as ATF3 p-eIF2α, when compared to untreated controls (**Figure 37**). Conversely, hIAPP-INS1E cells treated with chaperones (TUDCA, PBA, BiP or PDI) presented a decrease in ER stress by showing a reduction in ATF3 and p-eIF2α protein levels (**Figure 37**). Consisting with our previous results, BiP levels were not affected after exposure to HG+PA, confirming the results observed in **Figure 36A**. Together these data suggest that chaperones are able to ameliorate induced-ER stress in hIAPP-expressing β-cells; thus, improving chaperone capacity could be important in diminishing hIAPP-induced toxicity.

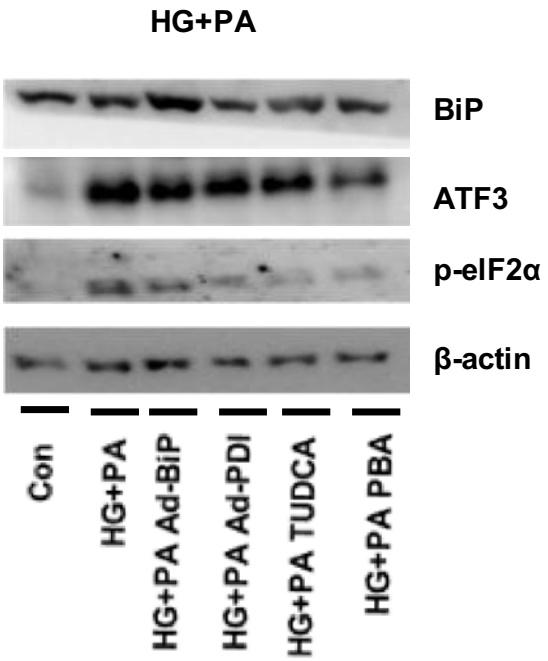


Figure 37. Chaperones decrease ER stress markers in hIAPP-INS1E cells. hIAPP-INS1E cells were treated with 25 mM of glucose and 400 µM palmitate (HG+PA) for 24 hours. BiP, ATF3, p-eIF2α and β-actin protein levels were analyzed by Western blot. Representative results from 3 individual experiments are shown.

5. CHAPERONE TREATMENT IMPROVES β-CELL FUNCTION BY INCREASING GLUCOSE-STIMULATED INSULIN SECRETION IN BASAL CONDITIONS

Our data suggests that chaperone treatment diminishes ER stress under thapsigargin and HG and PA conditions. In order to investigate whether chaperone amelioration in ER stress resulted in improved physiological outcome as measured by insulin secretion, we monitored the effect on glucose-stimulated insulin secretion in hIAPP-INS1E cells at basal conditions. Thus, we transduced hIAPP-INS1E cells and INS1E control cells with Ad-BiP, Ad-GFP and Ad-PDI/GFP or treated cells with chemical chaperones TUDCA and PBA. As shown in **Figure 38**, insulin release from INS1E cells at 16 mM glucose was increased in untreated or Ad-GFP treated cells as compared to 2.8 mM glucose. However, overexpression of hIAPP seems to alter glucose-stimulated insulin secretion when compared to INS1E control cells. In hIAPP-INS1E cells overexpressing BiP or treated with TUDCA and PBA, the levels of insulin secretion were higher compared with GFP-expressing or control cells at 16 mM glucose. Interestingly, overexpression of PDI increased the insulin levels to a greater extent than controls. Nevertheless, BiP and chemical treatment were able to restore and improve glucose-stimulated insulin secretion, although the insulin secretory response of control INS1E cells was not achieved.

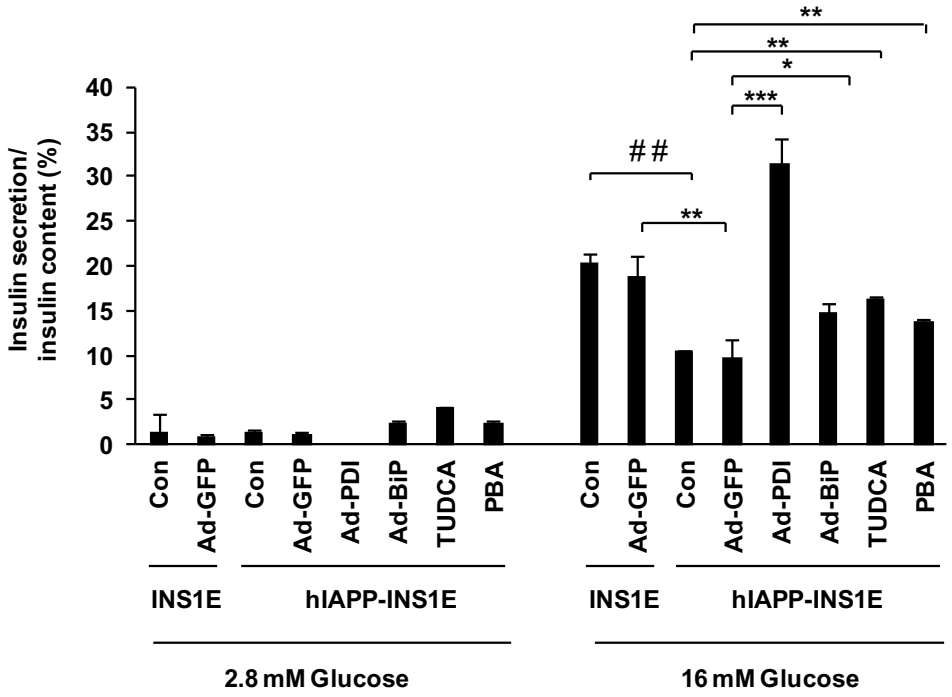


Figure 38. Chaperones ameliorate insulin secretion in hIAPP-INS1E cells in basal conditions. hIAPP-INS1E cells were transduced with Ad-GFP, Ad-BiP, Ad-PDI or treated with TUDCA or PBA for 24 hours. Glucose-stimulated insulin secretion was performed at low (2.8 mM) and high (16 mM) glucose expressed as % of insulin secretion/insulin content. INS1E control cells were used as a control (Con). Results are expressed as mean ± S.E.M from four independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ *vs* INS1E control, $^{\#\#}p < 0.001$ *vs* hIAPP-INS1E Control.

The improvement in insulin secretion in hIAPP-INS1E cells treated with BiP, TUDCA and PBA does not appear to be associated with a decrease in insulin content, since all groups had similar levels of insulin in lysates before and after exposure to glucose (**Figure 39**). However, PDI treatment of hIAPP-INS1E cells seems to have a detrimental effect on insulin content, suggesting that PDI may be increasing insulin secretion by degranulation of insulin vesicles (**Figure 39**).

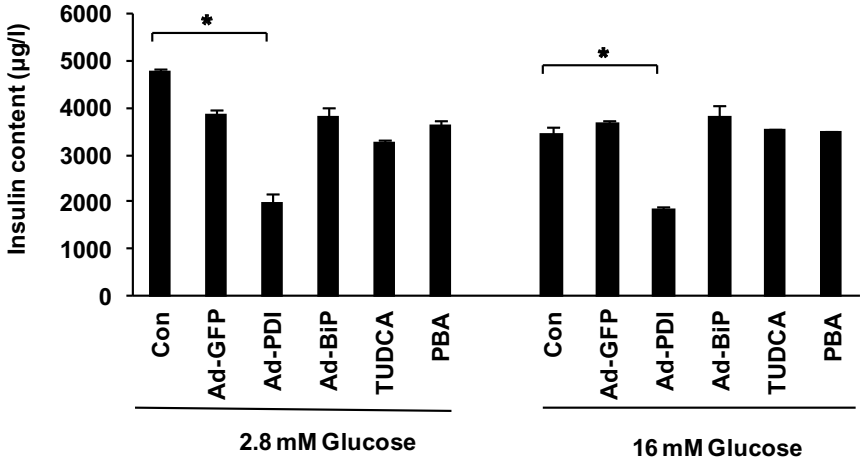


Figure 39. Insulin content is decrease in PDI-expressing cells. Total insulin content from hIAPP-INS1E cells transduced with Ad-GFP, Ad-BiP, Ad-PDI or treated with TUDCA or PBA for 24 hours was determined in lysates. Results are expressed as mean ± S.E.M from four independent experiments. *$p < 0.05$. No statistical differences were found in insulin content between Con and Ad-GFP, BiP, TUDCA and PBA.

6. CHAPERONE TREATMENT IMPROVES IMPAIRED β-CELL FUNCTION UNDER CONDITIONS OF HIGH GLUCOSE AND PALMITATE

In chapter 3.5 we have shown that HG+PA increased ER stress markers which may contribute to a detrimental effect on insulin secretion. In order to study the role of chaperones in insulin release under physiological stress conditions, hIAPP-INS1E and INS1E control cells were exposed to HG+PA for 24 hours in the presence of chaperones BiP, PDI, TUDCA and PBA. Glucose-stimulated insulin secretion (GSIS) experiments were performed at basal (2.8 mM) and stimulatory (16 mM) glucose concentrations. Under these experimental conditions, we observed that HG+PA exposure diminished 2.6-fold and 2-fold glucose-stimulated insulin release from hIAPP-INS1E cells at 16 mM glucose when compared to untreated INS1E and hIAPP-INS1E cells respectively. Surprisingly, treatment with BiP, PDI, TUDCA and PBA in conditions of HG+PA, was able to recover and normalize the values of insulin secretion in a similar way to untreated hIAPP-INS1E cells, suggesting that chaperone treatments were able to prevent β-cell dysfunction and maintain a

normal insulin secretory response (**Figure 40**). Insulin content of hIAPP-INS1E cells stimulated at 16 mM and 2.8 mM glucose was similar in all groups (**Table 4**) suggesting that differences observed in insulin secretion were due to the secretory capacity and not due to differences in insulin content. These results demonstrate that improving chaperone capacity can ameliorate β-cell function under stressful conditions.

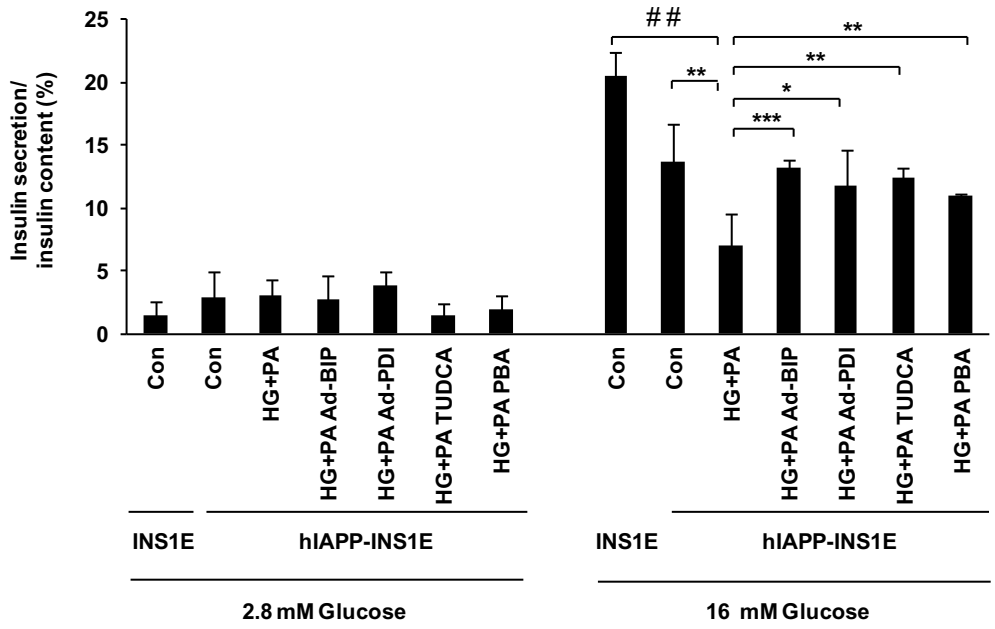


Figure 40. Chaperone administration prevents β-cell dysfunction under high-glucose and palmitate treatment. hIAPP-INS1E cells were transduced with Ad-BiP, Ad-PDI or treated with TUDCA or PBA for 24 hours. After 24 hours, cells were treated with 25 mM of glucose and palmitate (HG+PA). Glucose-stimulated insulin secretion was performed at low (2.8 mM) and high (16 mM) glucose using INS1E cells as control as expressed by % of insulin secretion/insulin content. Insulin levels were determined by ELISA. Results are expressed as mean ± S.E.M from three independent experiments. $^{\#\#}p < 0.05$ *vs* INS1E control, $*p < 0.05$, $**p < 0.01$, $***p< 0.001$ *vs.* hIAPP-INS1E cells treated with HG+PA. No statistical differences were found between Con and BiP, PDI, TUDCA and PBA.

Table 4. Effect of chaperone treatment in insulin secretion and insulin content in hIAPP-INS1E cells under HG+PA treatment.

	Culture conditions	Insulin secretion (ng/ml per 100000 cells) 2 mM	Insulin secretion (ng/ml per 100000 cells) 16 mM	Insulin content (ng/ml per 100000 cells) (16 mM)
	Con INS1E	0,50 ± 0,10	4,99 ± 0,69$^{\#\#}$	24,42 ± 1,78
hIAPP-INS1E	**Con**	0,62 ± 0,20	3,27 ± 0,65**	24,56 ± 4,38
	HG+PA	0,47 ± 0,09	1,33 ± 0,31	20,20 ± 3,74
	HG+PA Ad-BiP	0,31 ± 0,17	2,05 ± 0,23§	15,53 ± 4,36
	HG+PA Ad-PDI	0,50 ± 0,11	2,11 ± 0,51*	14,78 ± 2,44
	HG+PA TUDCA	0,35 ± 0,07	2,14 ± 0,16**	17,16 ± 2,28
	HG+PA PBA	0,44 ± 0,09	2,35 ± 0,13**	21,55 ± 2,26

Insulin secretion and content levels at 2 mM and 16 mM glucose from hIAPP-INS1E cells transduced with Ad-BiP and Ad-PDI or treated with TUDCA and PBA after exposure to HG+PA. $^{\#\#}p < 0.05$ *vs* Con INS1E, $*p < 0.05$, $**p < 0.01$, $^{\S}p < 0.001$ *vs* hIAPP-INS1E cells treated with HG+PA measured by ANOVA. A statistical difference of $***p < 0.001$ was found in all values of 2 mM *vs* 16 mM glucose as measured by student T-test.

AIM III

To study whether chaperones are able to ameliorate β-cell function and prevent amyloid formation in isolated hIAPP transgenic mouse islets

7. BiP AND PBA INCREASE INSULIN SECRETION IN WILD-TYPE MOUSE ISLETS

Our results have shown that chaperone treatment of hIAPP overexpressing β-cells was able to restore ER stress and insulin secretion in stressful condition, such as treatment with HG and PA. Once evaluated the chaperone treatment in pancreatic cell cultures lines we next evaluated the efficiency of the treatment in pancreatic mouse islets. Thus, to check whether adenovirus was able to transducer insulin-expressing cells, fresh isolated islets were transduced with green-fluorescence GFP and stained for insulin. Co-localization of GFP (green) with insulin (red) was observed using a dose of 20 MOI Ad-GFP (**Figure 41, lower panel**).

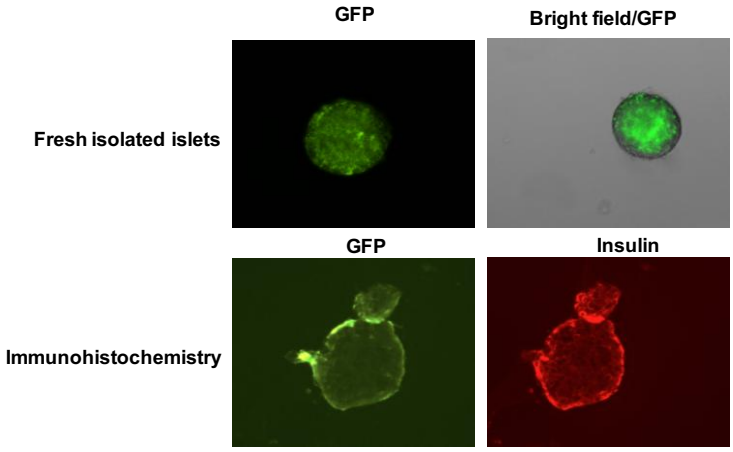


Figure 41. Pancreatic islets transduced with Ad-GFP at 20 MOI. Fluorescence images of Ad-GFP transduced with Ad-GFP 20 MOI of GFP (upper panel). Insulin staining of islets transduced with Ad-GFP 20 MOI (Lower panel, left column). Note co-localization of green (Ad-GFP transduced area) with insulin (red) demonstrating that adenovirus is able to transduce insulin-expressing cells. Representative images of islets are shown.

In order to determine the optimal adenoviral transduction dose, of Ad-BiP and Ad-PDI, isolated WT islets were transduced for 24 hours with different MOIs (10, 20, and 40) of Ad-BiP, Ad-PDI and Ad-GFP after an overnight incubation at 11 mM of glucose. To measure the insulin secretory capacity, islets were incubated for 60 minutes at 2 mM glucose, following stimulation at 16 mM glucose for 1 hour. As observed in **Figure 42**, islets transduced with 10 and 20 MOI of Ad-BiP enhanced insulin release when compared with WT islets transduced with Ad-GFP and control stimulated at 16 mM glucose. In contrast, 40 MOI of adenovirus showed a markedly reduction in

insulin secretion, suggesting a toxic effect leading to islet death. On the other hand, with PDI overexpression we were unable to detect any significant differences under 10 or 20 MOI of Ad-PDI when compared with control islets stimulated at 16 mM glucose, though the transduction with 40 MOI showed a strong augment of insulin release.

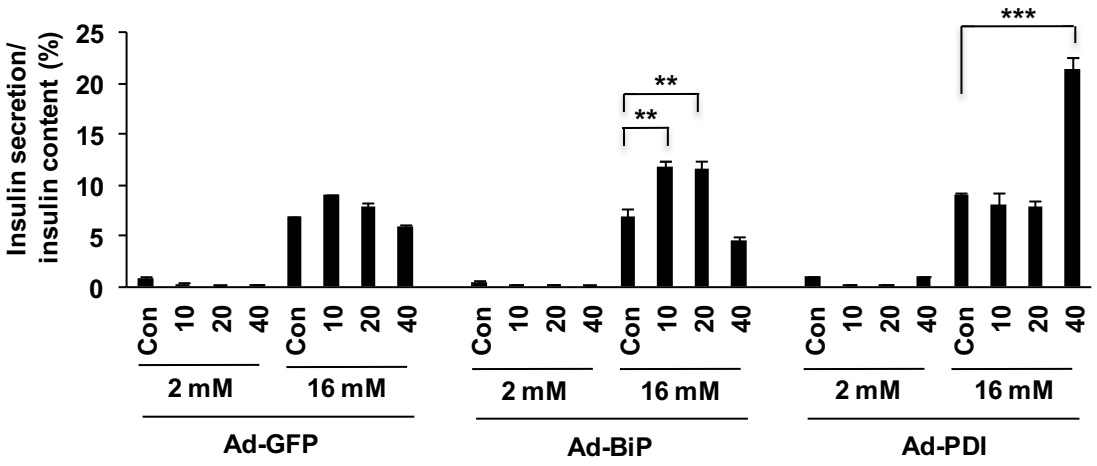


Figure 42. BiP increases insulin secretion in WT islets. WT islets were transduced with Ad-GFP, Ad-BiP, Ad-PDI for 24 hours. Glucose-stimulated insulin secretion was performed at low (2 mM) and high (16 mM) glucose. Results are expressed as % of insulin secretion/ insulin content. Non-transduced islets were used as a control (Con). Results are expressed as mean ± S.E.M from four independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ *vs* Con.

In addition, we observed that at a high dose (40 MOI) of Ad-BiP, Ad-GFP and Ad-PDI there was a significant alteration in insulin content when compared with control and lower doses of adenovirus suggesting that high adenoviral doses may lead to a toxicity effect (**Table 5**).

Table 5. Effect of different adenoviral transduction doses in insulin secretion and insulin content in WT islets.

Adenovirus (MOI)		Insulin secretion (ng/ml per islet)		Insulin content (ng/ml per islet) (16 mM)
		2 mM	16 mM	
Ad-GFP	0	0,27 ±0,03	7,22 ± 0,04	89,55 ± 1,07
	10	0,27 ± 0,02	7,60 ± 0,06	78,50 ± 1,52
	20	0,09 ± 0,01	7,40 ± 0,44	85,27 ± 2,52
	40	0,16 ± 0,02	5,19 ± 0,08	127,63 ± 4,06*
Ad-BiP	0	0,15 ± 0,04	6,62 ± 0,86	96,31 ± 9,86
	10	0,11 ± 0,01	7,14 ± 0,41**	85,75 ± 6,70
	20	0,16 ± 0,04	11,23 ± 0,72**	96,67 ± 3,03
	40	0,11 ± 0,03	6,03 ± 0,50	133,77 ± 0,74*
Ad-PDI	0	0,95 ± 0,07	8,16 ± 0,04	90,85 ± 1,67
	10	0,14 ± 0,01	6,67 ± 0,22	83,85 ± 15,88
	20	0,19 ± 0,00	5,32 ± 0,10	66,64 ± 2,43
	40	0,27 ± 0,01	5,41 ± 0,06§	22,91 ± 1,79*

Insulin secretion and content levels at 2 mM and 16 mM glucose from Ad-GFP, Ad-BiP and Ad-PDI transduced cells at indicated MOI. *$p < 0.05$, **$p < 0.01$, §$p < 0.001$ *vs* 0 MOI measured by ANOVA. A statistical difference of ***$p < 0.001$ was found in all values of 2 mM *vs* 16 mM glucose as measured by student T-test.

Once evaluated the effect of molecular chaperones in insulin secretion, we next examined whether chemical chaperones treatment with TUDCA and PBA affected insulin secretion in WT islets. As observed in **Figure 43**, 24 hours after treatment with both chemical chaperones, TUDCA showed no difference in insulin secretion output when compared with non-treated islets (Con) stimulated at 16 mM glucose. In contrast, islets treated with PBA had significantly enhanced glucose-stimulated insulin secretion compared with control.

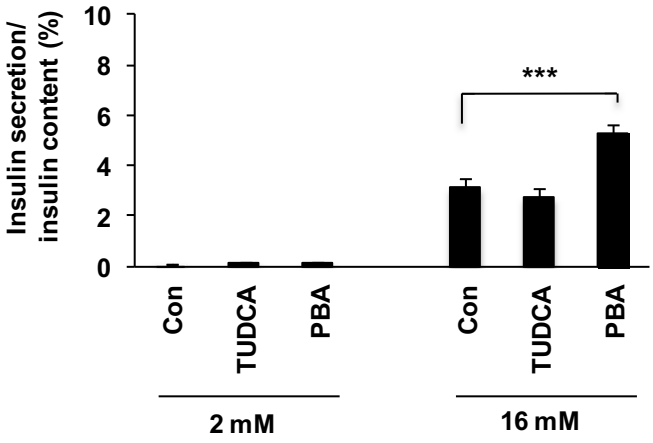


Figure 43. PBA increases insulin secretion in WT islets. WT islets were treated with TUDCA and PBA for 24 hours. Glucose-stimulated insulin secretion was performed at low (2 mM) and high (16 mM). Results are expressed as % of insulin secretion/ insulin content. Non-transduced islets were used as a control (Con). Results are expressed as mean ± S.E.M from 4 independent experiments. ***p < 0.001 *vs* Con. No statistical differences were found in secretion between Con and TUDCA.

We next measured insulin content for both chaperones. The insulin content in islets exposed to TUDCA or PBA showed no differences compared with control islets, suggesting that the increase insulin secretion is not a consequence of a reduce in insulin content (**Table 6**).

Table 6. Effect of chemical chaperone treatment in insulin secretion and insulin content in WT mouse islets.

Culture conditions	Insulin secretion (ng/ml per islet)		Insulin content (ng/ml per islet) (16 mM)
	2 mM	16 mM	
Con	1,49 ± 0,22	8,47 ± 3,42	253,23 ± 38,29
TUDCA	0,37 ± 0,13	7,72 ± 2,25	299,42 ± 60,34
PBA	1,32 ± 0.18	13,92 ± 0,13§	208,13 ± 27,27

Insulin secretion and content levels at 2 mM and 16 mM glucose from islets treated with TUDCA and PBA. §p < 0.001 *vs* Con measured by ANOVA. No statistical differences were found in secretion or content between Con and TUDCA. A statistical difference of ***p < 0.001 was found in all values of 2 mM *vs* 16 mM glucose as measured by student T-test.

8. BiP, PDI AND PBA INCREASE INSULIN SECRETION IN hIAPP TRANSGENIC MOUSE ISLETS

Having established that chaperones are able to increase insulin secretion in WT islets in basal conditions (11 mM), we next evaluated the effect of chaperones in hIAPP Tg mouse islets. The hIAPP Tg mouse is a mouse model of hIAPP overexpression specifically in β-cell, which gives us a good model to study the role of hIAPP *in vivo*. Thus, we overexpressed Ad-BiP, Ad-PDI and Ad-GFP by adenoviral transduction or treated islets with TUDCA or PBA for 24 hours. A significant increased in insulin secretion was observed in islets overexpressing the endogenous chaperone BiP and PDI at 16 mM glucose when compared with Ad-GFP hIAPP Tg islets (**Figure 44A**). On the other hand, as observed in **Figure 44B**, when islets were treated with PBA, insulin secretion was markedly increased when compared with control islets stimulated with 16 mM glucose. Instead, TUDCA was not able to improved insulin release in basal conditions.

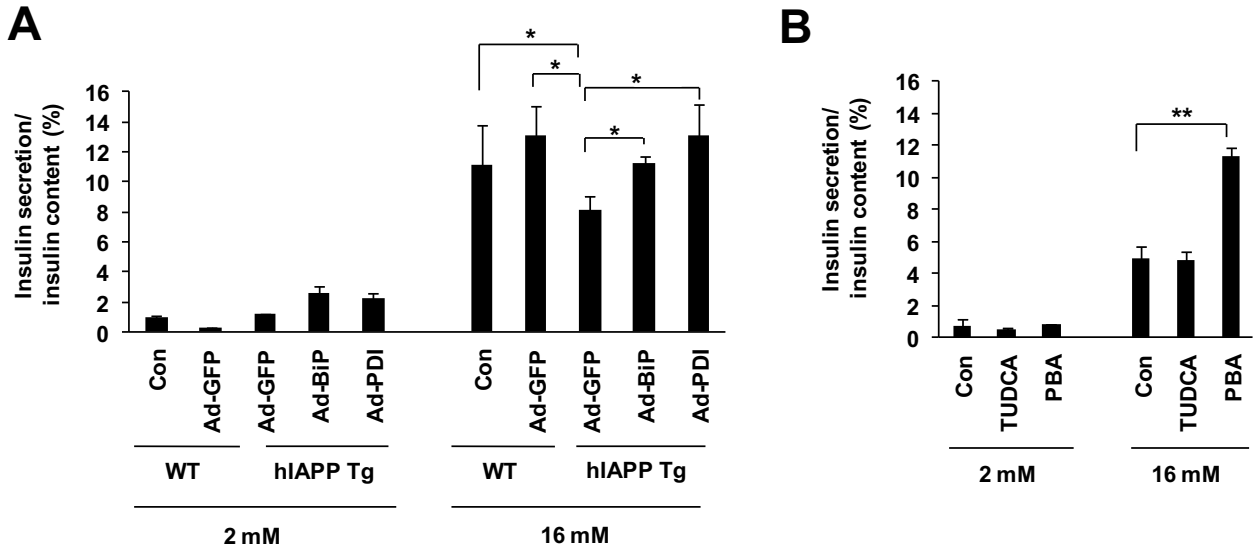


Figure 44. BiP, PDI and PBA increase insulin secretion in hIAPP transgenic mouse islets. hIAPP Tg mouse islets were transduced with 20 MOI of **A)** Ad-GFP, Ad-BiP and Ad-PDI or **B)** treated with TUDCA or PBA at 11 mM glucose for 24 hours. Glucose-stimulated insulin secretion was performed at low (2 mM) and high (16 mM) glucose. Insulin levels were determined by ELISA. Results are expressed as mean ± S.E.M from three independent experiments. *$p < 0.05$, **$p < 0.01$ *vs* hIAPP Tg Ad-GFP at or Con 16 mM glucose. No statistical difference was found between Con and TUDCA.

Insulin content was measured for each condition. We found that treatment with chaperones did not induce any significant alterations in insulin content levels when stimulated at 16 mM glucose, suggesting that increase insulin secretion is not a consequence of a reduction in insulin content (**Table 7**). Thus, enhancing chaperone capacity by increasing BiP and PDI levels or treating with PBA ameliorates β-cell function in basal conditions in hIAPP Tg mice.

Table 7. Effect of molecular and chemical chaperones in insulin secretion and insulin content in hIAPP Tg mouse islets.

	Insulin secretion (ng/ml per islet)		
Molecular chaperones	**2 mM**	**16 mM**	**Insulin content (ng/ml per islet) (16 mM)**
WT	0,76 ± 0,10	24,51 ± 5,49*	201,59 ± 15,33
WT Ad-GFP	0,27 ± 0,05	28,13 ± 3,26*	201,49 ± 35,22
hIAPP Tg Ad-GFP	0,95 ± 0,44	14,59 ± 2,65	187,12 ± 29,35
hIAPP Tg Ad-BiP	1,15 ± 0,46	23,17 ± 4,32*	193,91 ± 46,99
hIAPP Tg Ad-PDI	2,20 ± 0,35	21,45 ± 4,12*	159,00 ± 26,00
Chemical chaperones			
Con	1,40 ± 0,47	9,47 ± 2,26	174,10 ± 30,16
TUDCA	0,99 ± 0,13	10,73 ± 1,38	183,70 ± 23,32
PBA	2,45 ± 0,25	17,20 ± 88,75**	153,84 ± 37,04

Total insulin content from TUDCA and PBA was determined in lysates. Results are expressed as mean ± S.E.M from three independent experiments. Statistical *$p < 0.05$, **$p < 0.01$ *vs* hIAPP Tg Ad-GFP or Con at 16 mM glucose measured by ANOVA. No statistical difference was found in insulin content. A statistical difference of ***$p < 0.001$ was found in all values of 2 mM *vs* 16 mM glucose as measured by student T-test.

9. CHEMICAL CHAPERONES INCREASE INSULIN SECRETION FOLLOWING HG+PA TREATMENT IN hIAPP Tg MOUSE ISLETS

In order to study whether chaperone effects on ER stress are capable to ameliorate β-cell function in stressful conditions, we performed insulin secretion assays with the presence of HG+PA in hIAPP Tg islets treated with Ad-BiP, Ad-PDI, TUDCA and PBA for 24 hours. As expected, we observed that HG+PA exposure decreased insulin secretion in islets treated with HG+PA when compared to control islets at 16 mM stimulation glucose. As shown in **Figure 45**, overexpression of BiP and PDI by adenoviral transduction in cells exposed to HG+PA, markedly increased the levels of glucose-stimulated insulin secretion compared with hIAPP control islets cultured with HG+PA. Similarly, TUCDA and PBA treatment was able to increase insulin secretion when compared with hIAPP islets cultured with HG+PA and, at the same time restore the levels of insulin secretion reaching the values obtained in hIAPP control islets at 16 mM glucose. Unexpectedly, despite the significant increased in insulin secretion levels, overexpression of PDI showed decreased in insulin content, suggesting that PDI overexpression may have a toxic effect leading to degranulation of insulin vesicles. On the other hand, Ad-BiP, TUDCA and PBA presented similar levels of insulin in lysate before and after exposure to glucose (**Table 8**).

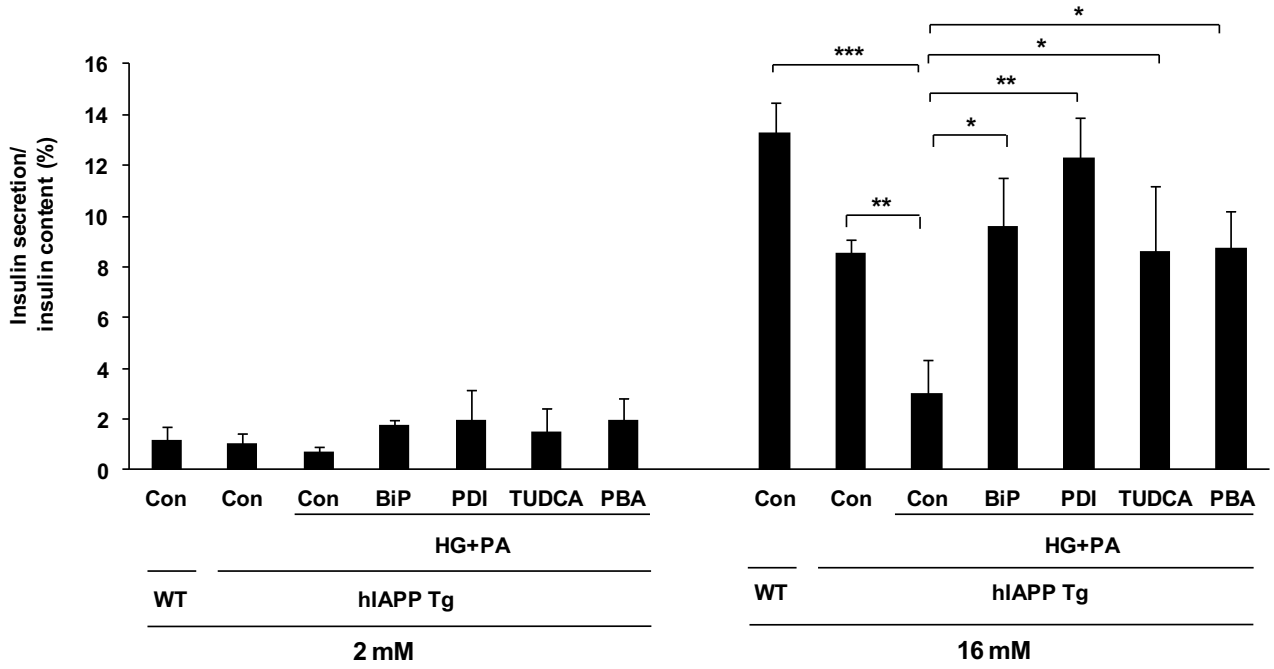


Figure 45. Chemical chaperones ameliorate β-cell function after high glucose and palmitate treatment. hIAPP Tg islets were treated with TUDCA or PBA for 24 hours and cultured with HG+PA for 24 hours more. Glucose-stimulated insulin secretion was performed at low (2 mM) and high (16 mM) glucose using INS1E cells as control. Results are expressed as % of insulin release/ insulin content. Insulin levels were determined by ELISA. Results are expressed as mean ± S.E.M from three independent experiments. *p < 0.05, ***p* < 0.01, ****p* < 0.001 *vs* Con HG+PA at 16 mM glucose.

Table 8. Effect of molecular and chemical chaperone treatment in insulin secretion and insulin content after HG+PA treatment in hIAPP Tg mouse islets.

Culture conditions		Insulin secretion (ng/ml per islet) 2 mM	Insulin secretion (ng/ml per islet) 16 mM	Insulin content (ng/ml per islet) (16 mM)
	WT	2,33 ± 0,34	22,74 ± 1,48§	169,56 ± 24,56
	hIAPP Tg	0,83 ± 0,16	13,26 ± 0,93**	159,63 ± 47,48
HG+PA	hIAPP Tg	0,98 ± 0,33	3,46 ± 1,92	95,42 ± 11,08
HG+PA	hIAPP Tg Ad-BiP	1,94 ± 0,57	17,83 ± 0,59*	182,72 ± 60,99
HG+PA	hIAPP Tg Ad-PDI	2,25 ± 0,26	16,23 ± 3,27**	132,19 ± 20,14
HG+PA	hIAPP Tg TUDCA	2,65 ± 0,30	16,92 ± 5,34*	170,41 ± 63,08
HG+PA	hIAPP Tg PBA	2,00 ± 1,02	16,45 ± 3,14*	178,13 ± 14,54

Results are expressed as mean ± S.E.M from three independent experiments. *p < 0.05, ***p* < 0.01, §*p* < 0.001 *vs* HG+PA hIAPP Tg measured by ANOVA. No statistical difference was found in insulin content. A statistical difference of ****p* < 0.001 was found in all values of 2 mM *vs* 16 mM glucose as measured by student T-test.

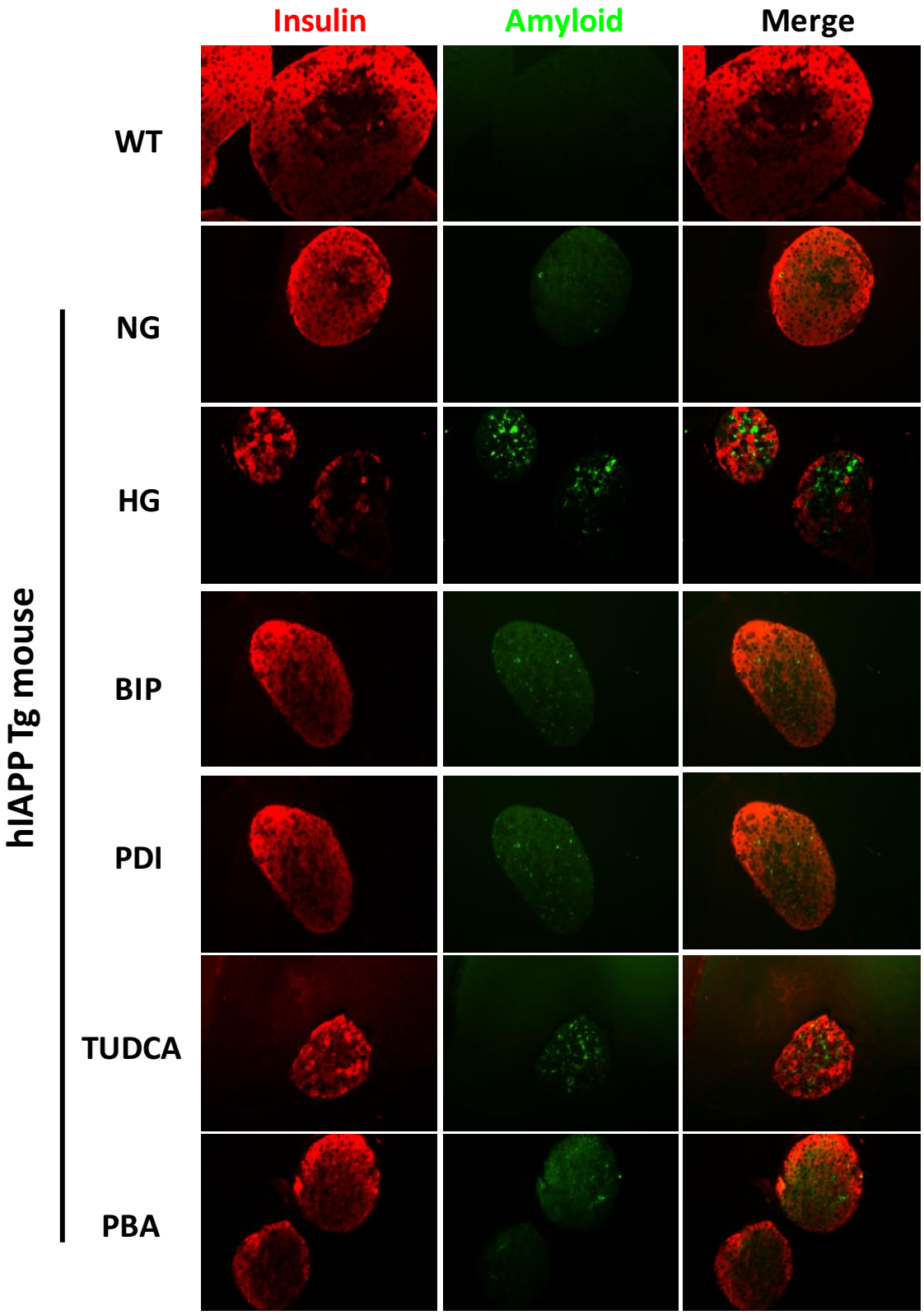
Together, our data suggests that either, molecular and chemical chaperone treatment can improve β-cell function under pathological conditions, suggesting that the observed effect of chaperones in ER stress correlates with an increase in insulin secretory capacity.

10. CHAPERONE TREATMENT PREVENTS AMYLOID FORMATION IN hIAPP Tg MOUSE ISLETS

We next investigated whether the decrease in ER stress and improvement of β-cell function observed in hIAPP-overexpressing β-cells correlates with a decrease in amyloid formation. While *in vivo* amyloid formation requires up to 10 months in hIAPP Tg mice, it can be rapidly induced by culturing isolated islets at high glucose concentrations (16 mM). Thus, non-transgenic and hIAPP Tg islets were cultured for 7 days at 16 mM of glucose (HG). Thioflavin S (thioS) staining showed

amyloid deposits in high glucose-cultured hIAPP Tg islets when compared with WT islets and hIAPP Tg islets cultured at 11 mM glucose (NG) (**Figure 46**) demonstrating that islet amyloid can be rapidly induced by culturing hIAPP Tg islets at HG concentrations *ex vivo.*

In order to investigate the role of molecular and chemical chaperones in amyloid formation in this model, hIAPP Tg mouse islets were transduced with 20 MOI of Ad-BiP and Ad-PDI or treated with chemical chaperones TUDCA and PBA. After 24 hours of transduction, islets were kept at 16 mM of glucose (HG) for 7 days. Remarkably, treatment of hIAPP Tg islets with molecular chaperones BiP and PDI resulted in a drastically decrease in amyloid formation when compared to non-treated islets cultured at HG. In addition, to determine whether chemical chaperones had the same effect as molecular chaperones on islets hIAPP Tg islets were treated with TUDCA and PBA under the same experiment conditions. Seven days after HG culture, amyloid formation was considerably inhibited under the treatment with PBA. On the other hand, TUDCA treatment showed a small (but not significant) reduction in amyloid formation (**Figure 46**).


Insulin
Amyloid
Merge
WT
NG
HG
BIP
PDI
TUDCA
PBA
hIAPP Tg mouse

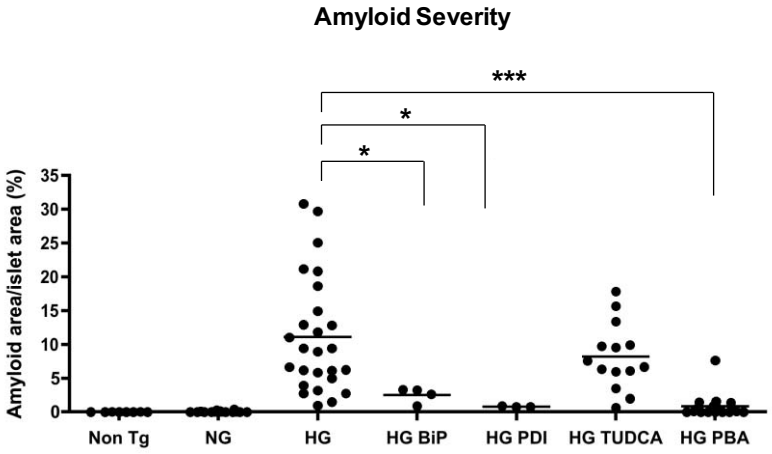


Figure 46. Decreased of amyloid formation after treatment with chaperones in hIAPP Tg mouse islets. Immunofluorescence staining for insulin (red) and amyloid (green) in isolated WT as controls and hIAPP Tg mouse islets cultured at 11 mM (NG) and 16 mM (HG) glucose for 7 days and treated with Ad-BiP, Ad-PDI, TUDCA and PBA (higher panel). Note amyloid deposits in HG treated islets. Amyloid area quantification of same experimental groups and normalized to insulin positive area (lower panel). Black dots represent individual islets. No statistical differences were found between HG and HG TUDCA. Results are expressed as mean ± S.E.M from two independent experiments. *p < 0.05, ***p < 0.001 *vs* HG.

In summary, our data suggests that treatment with chaperones not only ameliorates ER stress and insulin secretion, but also plays an important protective effect in the development of amyloid formation in a mouse model of amyloid deposition. Our results suggest that chaperone treatments may be used as a potential therapeutic approach to improve ER stress, β-cell function and amyloid deposition in T2D.

Chapter IV.
DISCUSSION

T2D, as a complex metabolic disease, is characterized by impaired insulin secretion with a progressive decline in β-cell mass and function. Amyloid deposits which often co-localize with cellular degeneration [74] have been strongly associated with the progressive loss of pancreatic β-cell. In recent years, the role of IAPP in the occurrence of T2D has gained much attention. Concomitantly, it has been proposed that overexpression of IAPP contributes to amyloid formation and development of T2D, a view proposed by transgenic and rat studies involving the overexpression of human IAPP in pancreatic β-cells [93]. In parallel, several studies implicate ER stress as an important cellular response triggered by IAPP leading to β-cell death [187]. Moreover, molecular chaperones have been described to be important for the regulation of ER signaling in response to ER stress and in decrease protein aggregation and amyloid deposition in neurodegenerative diseases [188-189]. Here we propose a novel approach to counteract hIAPP-induced ER stress as well as diminish amyloid deposits and, ultimately β-cell dysfunction through chaperone treatment in models of hIAPP overexpression. In our approach, both INS1E stable clones and hIAPP transgenic mouse islets were treated with chaperones after exposure to physiopathological conditions with the purpose to improve β-cell function by reducing ER stress as well as amyloid formation.

Several findings have revealed that ER stress in β-cell is associated with activation of UPR pathway, through activation of the three main components such as IRE1, PERK, and ATF6. Previous studies have shown that experimental manipulation of the UPR system in β-cells generally leads to attenuated function and cell death. For example, cells lacking IRE1α and XBP1 have defective ERAD as well as reduced secretory capacity and reduced cell survival [190]. Even more, β-cell specific XBP1 deficient mice elicited an impairment in β-cell proliferation, proinsulin processing, and insulin secretion along with a hyperactivation of IRE1 [191]. The loss of PERK expression in humans and mice has been associated with a failure to properly regulate the UPR response, underlying dysfunction in the ER stress and UPR pathways, which can result in increased cell death and diabetes [192]. Although PERK expression in adult β-cells does not appear to be required for maintaining β-cell function, mutations in PERK result in the elevation of ER stress markers, leading to a form of permanent neonatal diabetes in humans. Regarding the downstream target genes of PERK, loss-of-function of ATF3 gene have shown to reduce the effects of nutrients on apoptosis in INS1E cells [162] and deletion of CHOP reduced oxidative stress, improved glycemic control and

expanded β-cell mass in mice models with T2D [193]. Recent reports have tried to elucidate the role of ATF6, the third branch of the UPR, in β-cell function. For example, ATF6 knockdown in insulinoma cells showed a decrease in ER chaperones and induced cell apoptosis [194]. Similarly, ER stress-induced activation of ATF6 has been shown to suppress insulin gene expression. However, other studies have shown that ATF6 is not so relevant for β-cell death, when compared with PERK and IRE1 pathways [144, 195].

In the present work we used a model of rat pancreatic β-cell line, INS1E that was stably transfected with the hIAPP transgene (hIAPP-INS1E cells), the rIAPP gene (rIAPP-INS1E) or an empty vector (INS1E control cells). Despite the fact that hIAPP overexpression in this cell model played a detrimental role in insulin and IAPP secretion in response to glucose, it was described that cell death or expression of genes involved in ER stress was not affected in hIAPP-expressing cells when compared with control cells at basal conditions [75]. Thus, in order to initially characterize the induction of ER stress in our INS1E cell model we performed a time-dose response, treating cells with a known inducer of ER stress, thapsigargin. Thapsigargin induces ER stress by blocking the ability of the cell to pump Ca^{2+} into the ER. Since altered cytosolic Ca^{2+} has been implicated in many diseases including diabetes, this chemical inducer has been shown to be of interest for the understanding the pathogenesis of T2D. Consistent with previous studies [196-197] we showed that thapsigargin-induced ER stress was associated with an upregulation of *ATF3*, *sXBP1* and *CHOP* at time points 8 and 24 hours after thapsigargin (1 μM) treatment in INS1E control cells. Furthermore, the exposure of hIAPP-INS1E cells to different concentrations (0.25 - 1 μM) of thapsigargin for 24 hours resulted in apoptosis as shown with increase protein levels of CHOP and caspase 3 and decrease hIAPP expression, with the maximal effect at 1 μM when compared with lower doses, suggesting that thapsigargin induced many of the expected dysfunctional regulation in INS1E cells.

In the present study, we confirmed that the pathways involving ER stress response, such as CHOP, sXBP1 or ATF3 were not affected in stable hIAPP-INS1E cells when cultured at basal conditions (11 mM glucose). Furthermore we showed that 8 hours treatment with a non-lethal dose of thapsigargin (0.5 μM) induced a profound ER stress. In addition to chemical stress, induction we examine the ER stress in response to more physiological conditions, such as exposure to high glucose (HG) and palmitate (PA). Although the mechanism by which glucolipotoxicity cause

these alterations is not fully characterized, it has been reported that treatment of pancreatic β-cell with both HG and PA contributed to a lowering of ER Ca^{2+} levels [152]. Previous studies have shown that HG and PA are potent inducers of ER stress [150, 198]. Prentki et al. have shown β-cell death by apoptosis in both rat cell line and human islets caused by a synergistic effect of elevated concentrations of glucose and saturated FFA [158]. In line with this, with similar results as thapsigargin treatment, we showed that combination of HG and PA treatment potentiated ER stress markers such as *CHOP*, *ATF3* and *sXBP1* in hIAPP-expressing cells when compared to rIAPP or INS1E controls. These results suggest that the ER stress response was activated under glucolipotoxicity conditions, since these effects are not observed under conditions of HG and PA separately. The reason for this is not known, although it is possible that activation of UPR pathways for a period of 24 hours with HG or PA alone is not sufficient to induce a significant upregulation of gene expression changes or this might be a feature of this cell line, since INS1E cell cultured in the presence of PA for 24 hours induced an increase in ER stress markers [199]. Together, these data supports the view that hIAPP-INS1E cells are more sensitive to exogenous ER stress inducers. Thapsigargin or HG and PA treatment significantly potentiated the expression of ER stress markers in hIAPP-expressing cells, when compared to rIAPP- or non-expressing controls, suggesting that hIAPP, but not rIAPP, increased sensitivity to ER stress. The potentiated ER stress activation in hIAPP-INS1E cells can be explained due to the increased aggregation capacity of hIAPP or the formation of intracellular oligomers, which may further affect ER stress [75, 77, 200]. These results are in conformity with other studies in cells and mice, where exogenous stressors or β-cell secretagogues led to ER stress activation [81, 199, 201]. However, the role of hIAPP in ER stress induction in basal conditions still needs to be elucidated. In accordance with our studies, Hull et al. demonstrated that overexpression of hIAPP transgenic mice was not associated with significant increases in the expression of ER stress markers [202]. In contrast, some reports have shown that rodent overexpression of hIAPP activates ER stress-mediated apoptosis, leading to a reduction in β-cell mass [187]. These differences can be explained by the different levels of hIAPP expression or the use of different experimental models, such as transgenic mice, transgenic rats or INS1E stable cell lines.

We showed that thapsigargin-induced ER stress was associated with persistent protein upregulation of CHOP in hIAPP-INS1E cells. Similarly, HG and PA-induced ER stress was associated with upregulation of CHOP, suggesting that thapsigargin or HG and PA-induced ER stress and

apoptosis were dependent of CHOP. Thus, CHOP seems to be a primary mediator of ER stress-induced apoptosis in hIAPP-INS1E cells and seems to be activated upon prolonged ER stress signaling. Several reports showed that treatment with saturated fatty acids cause numerous alterations that can initiate apoptosis by different mechanisms, including reactive oxygen species, mitochondrial dysfunction, generation of ceramide or induction of CHOP and caspase 3 pathway [203-206]. In accordance with our results, deletion of CHOP has been shown to enhance β-cell function and mass in several models of β-cell stress and T2D. In addition, several studies demonstrate islets from CHOP knockout mice with fewer apoptotic cells and an increased expression of UPR genes delaying the onset and severity of the diabetic phenotype [193, 207]. More importantly, we showed that a decrease in CHOP expression preceded the reduction of cleaved caspase 3, which, on the other hand, was apparent after treatment with thapsigargin or HG and PA. Overall, these data suggest that CHOP plays a detrimental role in ER stress induction and that CHOP silencing may be a therapeutic approach to modulate β-cell function and survival in T2D.

Pancreatic β-cells face the challenge of increasing protein synthesis during acute or chronic stimulation leading to a burden on the ER, the organelle where insulin and IAPP synthesis and folding takes place. However, the large array of ER chaperones and enzymes within ER allow efficient folding, assembly and reducing the risks of aggregation. Therapeutic interventions that reduce ER stress have been studied in order to provide strategies for treating ER stress-related human diseases such as T2D [206]. Our first goal was to examine the contribution of ER stress in β-cell dysfunction in a model of hIAPP overexpression. It has been previously reported that overexpression of ER chaperones or the treatment with chemical chaperones could partially prevent β-cell dysfunction *in vitro* in INS1E cells [171]. Importantly, our studies strengthen the fact that activation of ER stress markers was reversed after treatment with chaperones. In thapsigargin or HG and PA treatment, the addition of chemical chaperones TUDCA and PBA diminished ER stress markers such as CHOP, ATF3 and p-eIF2α, highlighting the importance of such agents in preventing activation of ER-stress protein markers. These results are in agreement with a previous study in INS1E cells, in which TUDCA reduced apoptosis due to amelioration of ER stress by attenuation of CHOP and ATF4 expression [208]. Recently, PBA was reported to attenuate cytokine-induced ER stress and protecting against PA-induced cell death in INS1E cells [199]. We demonstrated similar effects after transduction with adenovirus encoding for endogenous chaperones BiP and PDI after

HG and PA treatment. However in the presence of thapsigargin, overexpression of BiP was not achievable due to a possible completed depletion of Ca^{2+} store levels by thapsigargin or a potential interference with thapsigargin and adenoviral transductions BiP and PDI. In accordance with our results, BiP overexpression has been shown to diminish apoptosis by attenuating the induction of CHOP in ER stress [136]. Combined, our results show that molecular and chemical chaperones are effective in reducing ER stress induced by physiopathological conditions, suggesting that chaperone treatment may be an approach to diminished hIAPP-exhacerbated ER stress.

Overexpression of IAPP has been associated with impaired β-cell function and development of T2D. Several studies obtained by our laboratory and others reported that IAPP overexpression contributes to the failure of insulin secretion in response to glucose stimulation [75, 209]. Thus, we aimed to determine whether chaperone amelioration of ER stress was able to restore GSIS. In agreement with previous results, hIAPP-INS1E cells showed a defect in insulin secretion when compared to INS1E control, confirming that this failure in insulin secretory capacity was specific for hIAPP overexpression in our cell model. Furthermore, the results obtained with GSIS in hIAPP-INS1E cells showed that treatment with chaperones BiP, TUDCA and PBA were able to ameliorate insulin secretory response under basal conditions (11 mM glucose). In contrast, PDI showed a marked increase in insulin secretion, accompanied with a significant decrease in insulin content. These results are consistent with a previous study showing that BiP can improve glucose-stimulated insulin secretion in INS1E cells exposed to HG. Furthermore, overexpression of PDI had no effect on insulin secretion. Although PDI is present in human islets [210] and has been shown to play an important role in sulphide bond formation and isomeration or protein degradation [211-213], its overexpression has been associated with induced ER stress resulting from accumulation of proinsulin in the ER [171], suggesting that PDI overexpression may have a detrimental effect that disrupts normal insulin processing. Thus, our experiments strongly suggested that treatment with BiP, TUDCA and PBA could ameliorate insulin secretory capacity in conditions of hIAPP overexpression.

It is now accepted that elevated HG levels are required to mediate the lipotoxic effects, including inhibition of glucose-stimulated insulin secretion [158, 214-215]. We could confirm that treatment of hIAPP-expressing cells with HG and PA for 24 hours significantly inhibited insulin secretion when compared to INS1E control cells. Since this reduction on insulin secretion was

mostly due to ER stress activation, we investigated whether molecular and chemical chaperones could reduce ER stress and improve the insulin output in our cell model. Thus, overexpression of BiP and PDI and treatment with TUDCA and PBA resulted in effective restoration of insulin secretory capacity. Our data suggest that improving chaperone capacity, we have been able to counteract the glucose-stimulated insulin secretion of hIAPP-INS1E cells.

We next focused on the effectiveness of chaperone treatment in isolated mouse islets. An increasing body of evidence suggests that chaperones play important protective effect in the decrease of protein aggregation and the pathophysiology of amyloid deposition in neurodegenerative diseases such as Alzheimer [188-189, 216]. In addition, overexpression of ER chaperone BiP in several cell systems such as HepG2 liver cells and mouse islets can protect cells against cell death caused by disturbances of ER homeostasis [164, 166-168]. Of interest, transgenic mice overexpressing the molecular chaperone BiP in β-cells are protected against the pathogenesis of obesity-induced T2D, maintaining β-cell function and improving glucose homeostasis [167]. In the current study, milder doses of Ad-BiP led to a significantly improve in glucose-stimulated insulin secretion in wild type (WT) islets at basal conditions as expected. In contrast, PDI overexpression showed no differences in insulin secretion at milder doses of adenovirus but an unexpectedly increment at high dose. Lately, recent reports indicate the beneficial effects of chemical/pharmacological chaperones in relieve ER stress and improving protein folding [163, 217]. Ozcan et al. have shown that chemical chaperones, such as PBA and TUDCA, reduce ER stress and restore glucose homeostasis in a mouse model of T2D [163]. Furthermore, PBA may provide health benefits by ameliorating insulin resistance and pancreatic β-cell dysfunction in obese subjects [184]. In this model, the oral chemical chaperone treatment of obese diabetic mice resulted in the normalization of hyperglycemia and restoration of peripheral insulin sensitivity, thus acting as a potential anti-diabetic agent [163]. Interestingly, our results show an improvement in insulin secretion after PBA treatment in WT islets under basal conditions. However, unlike PBA, TUDCA showed no effect in insulin release in WT islets, suggesting that TUDCA effects may only be seen under ER stress conditions. All together, these data highlight the ability of endogenous and chemical chaperones to alleviate ER stress in transgenic and obese mice models and strongly supports the ER stress-based mechanistic model of T2D demonstrating the feasibility of targeting ER function for therapeutic goals.

We therefore examined the consequence of hIAPP overexpression using hIAPP transgenic mouse islets (hIAPP Tg) and tested the effect of chaperones on insulin release at basal conditions. We found that hIAPP overexpression slightly decreased glucose-stimulated insulin secretion which is in accordance with our previous data [75] showing that hIAPP-overexpressing islets specifically induce a defect in insulin secretion. Though, insulin secretory capacity was partially rescued by BiP and PDI overexpression. The results obtained with chemical chaperones strongly support the same tendency as observed with WT islets. Despite measurable increase in insulin secretion under PBA treatment, there was no difference when cells were exposed to TUDCA treatment. Together, these data suggest that chaperone capacity of the ER may be involved in the mechanism of hIAPP-induced dysfunction in pancreatic mouse islets.

In the present study, we have demonstrated that isolated hIAPP Tg mice islets exposure to HG and PA is associated with a significant reduction in glucose-stimulated insulin secretion that was not observed in hIAPP Tg islets at basal conditions. This marked decrease in insulin release occurring under pathological conditions is in accordance with our previous results in hIAPP-INS1E cells, in which we observed a reduction in insulin secretion under the same conditions. These findings emphasize that glucolipotoxicity and the associated islet amyloid together result in impaired insulin secretion. A plausible explanation for this finding could rely on the imbalance between the delivery of nascent IAPP to ER and the capacity of the ER to fold, traffic, and process this amyloidogenic protein. In this current study we have shown a correlation of chaperone treatment and the resultant amelioration of insulin secretion. Herein, transduction of isolated hIAPP Tg islets with adenovirus encoding for BiP and PDI along with HG and PA for 24 hours not only restored but also improved insulin release. This result is in agreement with a previous study demonstrating that transgenic mice overexpressing BiP specifically in β-cells were protected against the injury of obesity-induced T2D, maintaining β-cell function and improving glucose homeostasis [167]. In a similar way, BiP overexpression has been shown to improve insulin sensitivity in ob/ob mice [218]. A similar result was obtained using the chemical chaperones TUDCA and PBA. These results are also consistent with those showed by Ozcan et al., in which chemical chaperones improved the *in vivo* responses to insulin in *ob/ob* mice.

The formation of amyloid fibrils is a hallmark of a variety of unrelated diseases. Despite its central public health importance, the mechanism of amyloid-related pathogenesis is not fully understood. Amyloid plaques occur in 90% of patients with T2D at autopsy [219], a finding that is uncommon in pancreata of non-diabetic humans [220]. Concomitantly, it has been proposed that overexpression of IAPP in pancreatic β-cells contributes to amyloid formation and disease severity with reduction in β-cell mass and development of hyperglycemia, a view proposed by transgenic mouse and rat studies [93, 220]. According with the literature, high glucose concentrations [221] exposure were found to be an important determinant of the extent of amyloid deposition [222]. Consistent with these reports, we have shown a rapid amyloid formation by culturing isolated hIAPP Tg islets at 16 mM glucose for 7 days, making this model particularly relevant to islet amyloid formation in humans, since amyloid deposits in both hIAPP Tg rodents and human islets are histologically analogous [38]. On the other hand, this model shows that hIAPP overexpression by itself is not sufficient to induce amyloid formation supporting the notion that stressful environment like hyperglycemia may be associated amyloidogenesis which is correlated with β-cell dysfunction [202, 223].

Chaperones are the primary regulators of the protein network and are known to facilitate protein folding, inhibit protein aggregation and promote disaggregation and clearance of misfolded aggregates inside cells. Efforts to understand the impact of chaperones on the formation of misfolded hIAPP have been considered a speculative approach to prevent or diminish amyloid formation. In our present work, we showed for the first time, the role of chaperones in amyloid formation in isolated pancreatic hIAPP Tg mouse islets. Here, we demonstrated that hIAPP Tg islets overexpressing ER chaperone BiP and PDI or treated with PBA markedly decrease the amount of amyloid formation when cultured at HG for 7 days. Few investigations have been performed in order to inhibit the aggregation of IAPP and for that reason it turns out to be a matter of considerable interest. Already a number of agents have been suggested as having this inhibitory effect including small peptides. In one of these few articles, Fraser and co-workers tested a series of hexapeptides, each overlapping with segment of IAPP [186]. When co-incubation with IAPP several of the peptides such as, GAILSST appeared to decrease the density of fiber formation. Additionally, some experimental works as well as computational or theoretical studies reveal that small molecules bind to- and stabilize a variety of protein fibrils formed by amyloidogenic proteins. In

fact, the fibrillation process of IAPP and its fragment (20-29) was found to be strongly affected by the presence of nanoparticles, which retard protein fibrillation as a function of the chemical surface properties of the nanoparticles. Moreover, recent studies demonstrated that ER chaperone BiP has direct interaction with amyloidogenic peptides [224] and has been shown to attenuate the formation of amyloid-like aggregates, suppressing the misfolding of hIAPP [164]. In addition, previous studies done by Butler et al, have shown that increased overload of hIAPP is correlated with an increased BiP overexpression due to activation of UPR response in β-cells of hIAPP transgenic mouse models [187].

Another promising approach is the use of pharmacological agents, such as orally active chemical chaperones, which can stabilize protein conformation, improve ER folding capacity and facilitate the trafficking of mutant proteins [163, 178, 184-185, 225-226]. In the current study we demonstrated that treatment with PBA decreased ER stress and ameliorated insulin secretion in hIAPP cells and hIAPP Tg mice. In addition, PBA dramatically reduced the level of amyloid severity, with similar results found in PBA-treated Alzheimer disease transgenic mouse (APPswe/PS1delta9), which showed a significantly decreased incidence and size of amyloid plaques through the cortex and hippocampus [227]. On the other hand and despite the fact that TUDCA decreases ER stress in hIAPP cells and prevents loss of insulin secretion upon treatment with HG and PA, in our mouse model of hIAPP overexpression, TUDCA slightly reduces amyloid formation, although more experiments need to be done. Nevertheless, in neurodegenerative disorders oral treatment with TUDCA has been shown to be effective in reducing β-amyloid deposits in brain of mice [228].

In summary, we showed that chaperones BiP, PDI, TUCDA and PBA decrease ER stress and improve GSIS in hIAPP cells. Additionally, we demonstrated that chaperones ameliorated glucose-stimulated insulin secretion in hIAPP Tg islets and we provide for the first time evidences that chaperones are able to prevent amyloid formation after incubation of islets with HG for 7 days. Although the role of chaperones in preventing hIAPP aggregation in islets is still poorly investigated our results support for the first time the investigation of endogenous and chemical chaperones as potential therapeutic strategies to improve ER stress and insulin secretion as well as limit the damaging amyloid observed in T2D patients. Finally, genuine understanding of the mechanism that leads to the formation of amyloid fibrils, its inhibition and the effect on the pathogenesis process

would be important for the basic understanding of the phenomenon and at the same time for development of future therapeutic approaches.

Chapter V.
CONCLUSIONS

In conclusion, our work enabled a better understanding of how ER stress affects β-cell function in a context of hIAPP overexpression:

1) hIAPP-INS1E cells were more sensitive to ER inducers than rIAPP and INS1E control cells.

2) ER stress induction was dependent on CHOP activation, protecting from induced ER stress and apoptosis in a cell model of hIAPP overexpression.

3) Molecular and chemical chaperones were able to ameliorate induced-ER stress in hIAPP-expressing β-cells.

4) Chaperone treatment led to an improvement in glucose-stimulated insulin secretion in hIAPP-INS1E cells in both basal and stressing conditions.

5) BiP overexpression and PBA treatment potentiated the insulin secretory capacity in WT mouse islets in basal conditions.

6) BiP and PDI overexpression and PBA treatment increased insulin secretion in hIAPP Tg mouse islets in basal conditions.

7) BiP, PDI, TUDCA and PBA improved insulin secretion under high glucose and palmitate treatment in hIAPP Tg mouse islets.

8) Chaperone treatment reduced amyloid severity in hIAPP Tg islets exposure at 16 mM glucose for 7 days.

9) Therefore, this study confirmed the role of hIAPP overexpression in triggering ER stress response and showed the first new protective mechanism against the development of amyloid formation through chaperone treatment under physiopathological conditions in a mouse model of amyloid deposition.

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PUBLICATIONS

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Chaperones Ameliorate Beta Cell Dysfunction Associated with Human Islet Amyloid Polypeptide Overexpression

Lisa Cadavez[1,2☯], Joel Montane[1,2☯], Gema Alcarraz-Vizán[1,2], Montse Visa[1,2], Laia Vidal-Fàbrega[1], Joan-Marc Servitja[1,2], Anna Novials[1,2]*

1 Diabetes and Obesity Research Laboratory, Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Hospital Clínic de Barcelona, Spain, **2** Centro de Investigación Biomédica en Red de Diabetes y Enfermedades Metabólicas Asociadas (CIBERDEM), Barcelona, Spain

Abstract

In type 2 diabetes, beta-cell dysfunction is thought to be due to several causes, one being the formation of toxic protein aggregates called islet amyloid, formed by accumulations of misfolded human islet amyloid polypeptide (hIAPP). The process of hIAPP misfolding and aggregation is one of the factors that may activate the unfolded protein response (UPR), perturbing endoplasmic reticulum (ER) homeostasis. Molecular chaperones have been described to be important in regulating ER response to ER stress. In the present work, we evaluate the role of chaperones in a stressed cellular model of hIAPP overexpression. A rat pancreatic beta-cell line expressing hIAPP exposed to thapsigargin or treated with high glucose and palmitic acid, both of which are known ER stress inducers, showed an increase in ER stress genes when compared to INS1E cells expressing rat IAPP or INS1E control cells. Treatment with molecular chaperone glucose-regulated protein 78 kDa (GRP78, also known as BiP) or protein disulfite isomerase (PDI), and chemical chaperones taurine-conjugated ursodeoxycholic acid (TUDCA) or 4-phenylbutyrate (PBA), alleviated ER stress and increased insulin secretion in hIAPP-expressing cells. Our results suggest that the overexpression of hIAPP induces a stronger response of ER stress markers. Moreover, endogenous and chemical chaperones are able to ameliorate induced ER stress and increase insulin secretion, suggesting that improving chaperone capacity can play an important role in improving beta-cell function in type 2 diabetes.

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* Email: anovials@clinic.ub.es

☯ These authors contributed equally to this work.

Introduction

One of the major pathological features of the pancreas in type 2 diabetes (T2D) is the presence of islet amyloid deposits, found in more than 80% of patients at autopsy [1]. These deposits are implicated in the process of β-cell deterioration and reduction in beta-cell mass and involve islet amyloid polypeptide (IAPP) aggregation of monomers into oligomers, fibrils and, ultimately, mature amyloid deposits [2,3,4,5].

The endoplasmic reticulum (ER) is the site of several important functions, including the synthesis, folding and maturation of secreted proteins. The pancreatic beta-cell has an extremely developed ER enabling the secretion of proteins such as insulin or IAPP [6,7]. However, the accumulation of misfolded proteins can alter ER homeostasis [7]. As a consequence, cells activate a succession of signal transduction cascades termed unfolded protein response (UPR), which may trigger inflammation and, ultimately, cell death [7,8,9,10].

To address the problem of misfolded proteins, cells have developed complex mechanisms that assist correct folding in the ER. Folding factors termed chaperones bind unfolded secretory proteins and prevent them from misfolding and aggregating [11]. Previous studies have shown that beta-cell-specific overexpression of glucose-regulated protein 78 kDa (GRP78, also known as BiP) can protect transgenic mice from disturbances in ER homeostasis, such as glucose intolerance and insulin resistance, induced by high-fat diet treatment [12]. In addition, adenoviral BiP overexpression is able to reduce ER stress [13] and reverse hyperglycemia- and hyperlipidemia- induced insulin synthesis and secretion *in vitro* [14]. Similarly, protein disulfide isomerase (PDI), which catalyzes the formation and breakage of disulfide bonds, has been shown to have important benefits as a chaperone [15,16] and to play a key role in the ER-associated protein degradation process [17]. Recent reports indicate beneficial effects of chemical or pharmacological chaperones, such as taurine-conjugated ursodeoxycholic acid (TUDCA) or 4-phenylbutyrate (PBA), in relieving ER stress and improving protein folding [18,19,20], particularly through oral treatment in rodent models of obesity and T2D [18]. Moreover, increasing evidence in neurodegenerative disorders points to a role of chaperones in preventing the

protein aggregation leading to attenuation in ER stress [21,22,23]. Since this is a common feature also observed in T2D, accumulation of misfolded human IAPP (hIAPP) may be responsible for the progression of the disease.

Our group and others have previously demonstrated that extracellular hIAPP aggregation is associated with ER stress responses in mouse beta-cells [24,25]. Furthermore, we have detected toxic intracellular aggregates in a rat pancreatic beta-cell line overexpressing hIAPP, which lead to a defective insulin and IAPP secretion in response to glucose [26]. Nevertheless, the role of hIAPP overexpression and ER stress induction has not been fully clarified.

The main objective of this work is to elucidate the role of endogenously produced hIAPP in ER stress induction in a rat pancreatic beta-cell line. In addition, we aim to study whether chaperones can ameliorate exogenously-induced ER stress in order to identify new targets for preventing the loss of beta-cell function in T2D. In the present study, we show that hIAPP potentiates induced ER stress in hIAPP-expressing beta cells. Moreover, the overexpression of molecular chaperones, or treatment with chemical chaperones, is able to ameliorate induced-ER stress and restore insulin secretion. Our results suggest that improving chaperone capacity may be important for diminishing stress in hIAPP-expressing cells, which ultimately can impact the formation of amyloid deposits in T2D.

Materials and Methods

Cell culture and treatments

A rat pancreatic beta-cell line INS1E overexpressing human and rat IAPP was previously established by our laboratory [26]. Briefly, these cells were stably transfected with hIAPP cDNA (hIAPP-INS1E cells), rat IAPP (rIAPP-INS1E) or an empty vector (INS1E control) under the cytomegalovirus promoter (CMV). Cells were maintained in complete RPMI 1640 (Sigma) supplemented with 1 mM sodium pyruvate (Thermo Scientific), 10 mM HEPES (Thermo Scientific), 10% Fetal Bovine Serum, 2 mM L-glutamine, 5 μM beta-mercaptoethanol, 200 μg/ml geneticin (Gibco) and 100 units/ml of penicillin and 100 μg/ml streptomycin at 37°C with 5% CO_2. Palmitic acid (400 μM, Sigma), combined with 25 mM of glucose, was added to the cells by conjugating with 1% (w/v) of albumin from bovine serum (BSA, Sigma). Thapsigargin (Sigma) was diluted in DMSO and used at a final concentration of 0.5 μM. PBA (Sigma) was dissolved in PBS and used at 2.5 mM. TUDCA (Calbiochem) was diluted in water and used at 200 μM.

Adenoviral transduction

The recombinant adenoviruses encoding for BiP and PDI/GFP under the CMV promoter were produced following earlier protocols [14,27]. hIAPP-, rIAPP- and control INS1E cells were transduced with 20 MOI of Ad/CMV-BiP, Ad/CMV-PDI or Ad/CMV-GFP, and incubated at 37°C and 5% CO2 for 2 hours. Cells were then washed with PBS and incubated at 37°C and 5% CO2 for 24 hours in fresh media RPMI 1640 prior to further treatment.

Cell viability assay

LIVE/DEAD Viability/Cytotoxicity Kit (Invitrogen) was performed following the manufacturer's instructions. Briefly, adenoviral transduced cells were cultured in the presence of 20 MOI of Ad-BiP. Cells were washed with PBS, and combined LIVE/DEAD assay reagents were applied for 30 min at room temperature. Images were captured using an Olympus Bx61 microscope and In Vivo or DP controller software. Thapsigargin treatment for 24 hours was used as positive control.

Small interfering RNA transfection

Knockdown of CCAAT/enhancer-binding protein (C/EBP) homologous protein (CHOP) expression in cells was performed using 20 μM of small interfering RNA (Invitrogen) or 20 μM of scramble siRNA (Applied Biosystems) as a control using Metafecten Pro (Biontex), according to the manufacturer's instructions. At six hours, the medium was changed to complete RPMI. Twenty-four hours after transfection, cells were treated with either, thapsigargin or 25 mM glucose and BSA-coupled palmitic acid (400 μM) for another 24 hours.

RNA isolation and Real-Time qPCR

Total RNA from cells were extracted using TRIZOL reagent (Invitrogen) following the manufacturer's instructions. Reverse transcription was performed with 0.5–1 μg of total RNA using Superscript III (Invitrogen), following the manufacturer's instructions. Real-Time PCR (RT-PCR) was carried in duplicates with 2 μg of transcribed cDNA and MESA Green qPCR MasterMix Plus FOR SYBR (Eurogentec) in a LightCycler 480 II sequence detection system (Roche Applied Science). PCR products were verified through dissociation curve analysis using SDS software (Roche Applied Science). Expression levels were normalized to TATA box-binding protein 1 (Tbp1) mRNA and represented in arbitrary units. The sets of primers used in these experiments can be seen in Table 1.

Western blot analysis

Cultured cells were washed in PBS and lysed in an ice-cold lysis buffer (50 mmol/l Tris Ph 7.5, 5 mmol/l EDTA, 150 mmol/ NaCl, 1% Triton X-100, 10 mmol/l sodium phosphate, 10 mmol/l sodium fluoride, 10 mmol/l and 10% of proteases inhibitors) for 20 min on ice, followed by centrifugation at 12′000×g for 20 min at 4°C. Protein concentration in the supernatant was determined using Bio-Rad Protein Assay kits (Bio-Rad), following the manufacturer's instructions. Protein samples (20–30 μg) were resolved by SDS-PAGE and transferred to PVDF membranes (PerkinElmer Life Sciences). Membranes were blocked for 2 hours with 5% skim milk or 5% BSA and incubated overnight at 4°C in primary antibodies: BiP/GRP78 (1:1000, Santa Cruz), peIF2α (1:1000, Cell Signaling), ATF3 (1:1000, Santa Cruz), CHOP (1:1000, Cell Signaling) and anti-β-actin antibody (1:1000). Membranes were washed with TBST and incubated with horseradish peroxidase-conjugated secondary antibodies (GE Healthcare) for 2 hours. Immunoreactive protein bands were developed with an ECL chemiluminescence reagents kit (Pierce). Changes in protein levels were evaluated by Quantity One software (Bio-Rad laboratories).

Glucose Stimulated Insulin Secretion (GSIS) Assay

hIAPP-INS1E cells were transduced overnight with 20 MOI of Ad-GFP, Ad-BiP, Ad-PDI, TUDCA or PBA. Cells were preincubated in a Krebs-Ringer bicarbonate buffer (KRB) containing 140 mM NaCl, 4.5 mM KCl, 2.5 mM CaCl2, 1 mM MgCl2.6H2O, 20 mM HEPES, pH 7.4, and supplemented with 0.1% BSA and 2.8 mM glucose for 30 min at 37°C with 5% CO2, following by stimulation with 2.8 mM or 16.7 mM glucose KRB for 1 hour at 37°C. Supernatant was recovered and cells were lysed in 500 μl of acid-ethanol solution to measure insulin content. Insulin levels and contents were determined by insulin ELISA (Mercodia), according to the manufacturer's protocol.

Table 1. List of primer sequences used in qRT-PCR experiments.

Primer	(5′----3′)	Species	Gene ID
ATF3 Forward	GCTGGAGTCAGTCACCATCA	Rat	Atf3
ATF3 Reverse	ACACTTGGCAGCAGCAA	Rat	Atf3
CHOP Forward	CCAGCAGAGGTCACAAGCAC	Rat	Ddit3
CHOP Reverse	CGCACTGACCACTCTGTTTC	Rat	Ddit3
Spliced XBP1 Forward	GAGTCCGCAGCAGGTG	Rat	Xbp1
Spliced XBP1 Reverse	GCGTCAGAATCCATCCATGGGA	Rat	Xbp1
TBP1 Forward	GAGATCACCCTGCAGCATCA	Rat	Tbp
TBP1 Reverse	GCAGTGCCGCCCAAGTAG	Rat	Tbp
BiP/GRP78 Forward	TGCAGCAGGACATCAAGTTC	Rat	Hspa5
BiP/GRP78 Reverse	AAAGAAGACCCCGTTTACAG	Rat	Hspa5
ATF3 Forward	TCGGATGTCCTCTGCGCTGGA	Mouse	Atf3
ATF3 Reverse	CTGACTCTTTCTGCAGGCACTCTGT	Mouse	Atf3
CHOP Forward	AAGATGAGCGGGTGGCAGCG	Mouse	Ddit3
CHOP Reverse	GCACGTGGACCAGGTTCTGCT	Mouse	Ddit3
Spliced XBP1 Forward	GAACCAGGAGTTAAGAACACG	Mouse	Xbp1
Spliced XBP1 Reverse	AGGCAACAGTGTCAGAGTCC	Mouse	Xbp1
TBP1 Forward	ACCCTTCACCAATGACTCCTATG	Mouse	Tbp
TBP1 Reverse	ATGATGACTGCAGCAAATCGC	Mouse	Tbp
BiP/GRP78 Forward	TGCAGCAGGACATCAAGTTC	Mouse	Hspa5
BiP/GRP78 Reverse	TACGCCTCAGCAGTCTCCTT	Mouse	Hspa5

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Immunohistochemistry

hIAPP-INS1E cells were fixed in 4% paraformaldehyde for 10 minutes. After blocking with PBS in 0.2% FBS cells for 1 hour, cells were immunostained using guinea pig anti-insulin (Dako) and rabbit anti-caspase 3 antibodies (Cell signaling), and goat anti-guinea pig Alexa Fluor 594 and goat anti-rabbit Alexa Fluor 594 (Molecular Probes) as secondary antibodies.

Statistical analysis

Statistical analysis between two groups was performed using Student's two-tailed t test and differences among more than two groups were carried out by ANOVA followed by Tukey test. Differences were considered significant when $*p<0.05$. Data in bar graphs are represented as mean ± SEM.

Results

ER stress-induced apoptosis in hIAPP-INS1E cells

To examine whether hIAPP overexpression potentiates ER stress in beta-cells, our group established a model in which a rat pancreatic beta-cell line, INS1E, was stably transfected with either hIAPP (hIAPP-INS1E cells), rIAPP (rIAPP-INS1E) or an empty vector (INS1E control cells) [26]. After treatment with chemical ER stress inducer thapsigargin at 1 μM for 8 and 24 hours, INS1E cells increased expression levels of ER stress genes such as activating transcription factor 3 (ATF3), spliced X-box binding protein 1 (sXPB1) and CCAAT/enhancer-binding protein (C/EBP) homologous protein (CHOP) (Figure 1A). Furthermore, 1 μM thapsigargin treatment for 24 hours did not increase the protein expression of CHOP or cleaved caspase 3 and did not affect the expression levels of insulin or IAPP (Figure 1B, left panel). However, treatment of hIAPP-INS1E cells with 1 μM of thapsigargin for 8 hours induced ER stress and severe apoptosis, by activation of CHOP and cleaved caspase 3 (Figure 1B, right panel). In addition, thapsigargin treatment decreased insulin and hIAPP production (Figure 1B, right panel), indicating that 1 μM of thapsigargin was associated with high toxicity in hIAPP-INS1E cells. Thus, to determine the optimal thapsigargin doses in hIAPP-expressing cells, we performed a dose/response experiment (0.25, 0.5 and 1 μM) at 8 and 24 hours. hIAPP-INS1E cells showed a high activation of ER stress marker CHOP and effector cleaved caspase 3 at 24 hours (Figure 1C). Furthermore, hIAPP-INS1E cells lost hIAPP expression, suggesting that either, 0.5 or 1 μM thapsigargin at 24 hours proved to be lethal in these cells (Figure 1C). Nevertheless, the dose of 0.5 μM of thapsigargin at 8 hours showed a mild activation of CHOP, absence of cleaved caspase 3 and unaffected hIAPP expression. Thus, a dose of 0.5 μM for 8 hours was chosen for further experiments based on mild ER stress induction in the absence of apoptosis.

hIAPP-INS1E cells are more sensitive to ER stress inducers

At 11 mM of glucose, hIAPP-INS1E cells did not show changes in ER stress genes, such as CHOP, spliced XBP1 (sXBP1) or ATF3, when compared to rIAPP-INS1E or INS1E control cells (Figure 2A). When cells were exposed to 0.5 μM thapsigargin for 8 hours, the expression of CHOP and ATF3 was significantly higher in hIAPP-INS1E when compared to rIAPP-INS1E or INS1E control cells (Figure 2B). In order to investigate the effect of physiological ER stress inducers, hIAPP-INS1E, rIAPP-INS1E and INS1E cells were cultured at 25 mM glucose for 8 h. However, hIAPP-INS1E cells did not show changes in protein or gene expression as compared to basal 11 mM glucose (data not shown). Conversely, when hIAPP-INS1E cells were treated with 25 mM of glucose together with 400 μM palmitic acid (PA), ER stress markers such as CHOP, ATF3 and sXBP1 significantly increased mRNA levels (Figure 2C) when compared to other

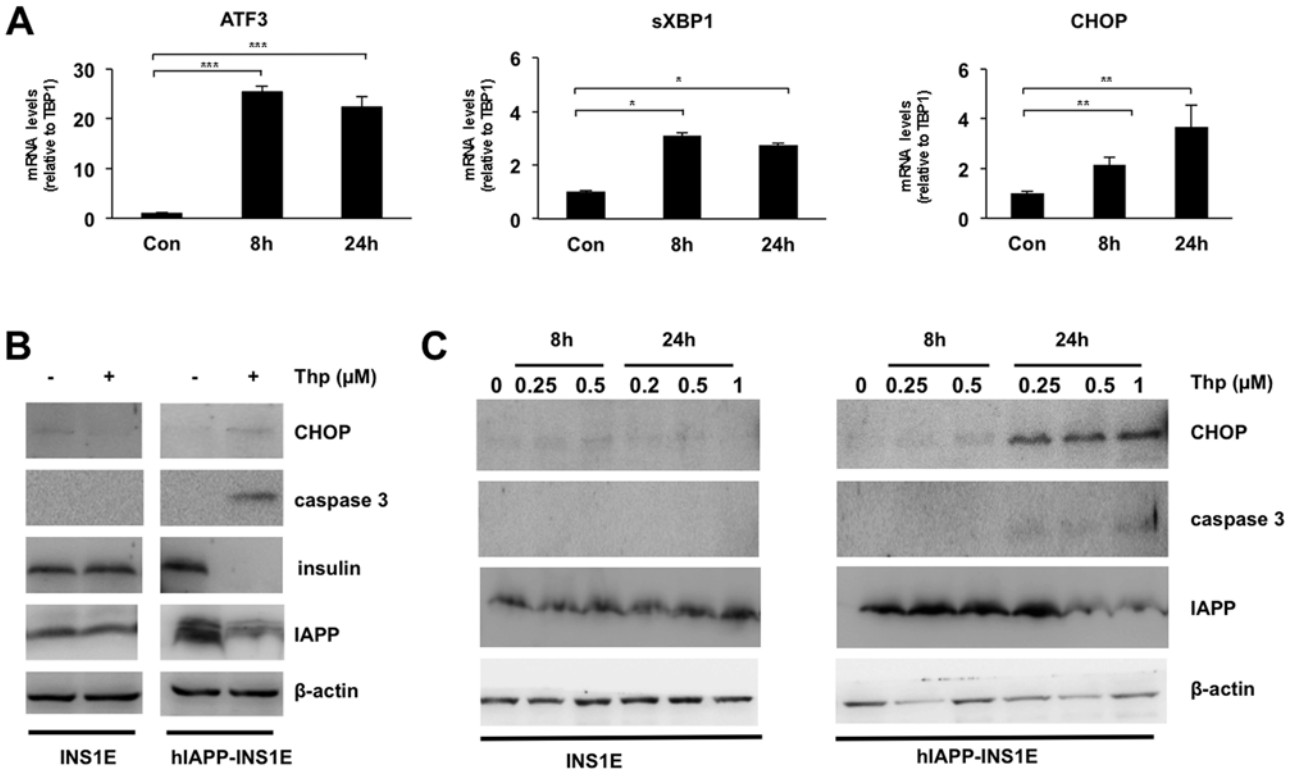

Figure 1. Thapsigargin induces ER stress and apoptosis in hIAPP-INS1E cells. A) INS1E control cells were cultured at 11 mM glucose and treated with 1 μM of thapsigargin for 8 and 24 hours. mRNA levels of ER stress markers ATF3, sXBP1 and CHOP were quantified by Real-Time PCR. *p<0.05, **p<0.01 and ***p<0.001 versus control cells at time 0. Results are expressed s mean ± S.E.M from five independent experiments. B) INS1E and hIAPP cells were exposed to 1 μM thapsigargin for 24 hours. Expression levels of CHOP, cleaved caspase 3, insulin, IAPP and β-actin were determined by Western blot. C) INS1E and hIAPP-INS1E control cells were exposed to 0.25, 0.5 and 1 μM of thapsigargin (Thp) for 8 and 24 hours. Protein levels of CHOP, cleaved caspase 3, IAPP and β-actin were determined by Western blot (n = 3). Representative Western blotting images are shown from 2 to 3 independent experiments.
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controls. These results indicate that the overexpression of hIAPP is sensed by the ER and triggers the activation of the UPR pathway, making hIAPP-INS1E cells more sensitive to ER stress than rIAPP-INSE or INS1E control.

CHOP inhibition protects from induced ER stress and apoptosis in hIAPP-expressing INS1E cells

We tested whether CHOP was required for the high glucose and palmitic acid-induced ER stress in hIAPP-INS1E cells. Small interfering RNA siCHOP was used to knockdown the expression of CHOP in hIAPP-INS1E cells previously treated for 24 hours with either thapsigargin or high glucose and palmitic acid. Scrambled siRNA was used as a control. As expected, treatment with both thapsigargin or high glucose and palmitic acid induced the expression of CHOP and downstream caspase 3. Upstream ATF3 protein levels were not affected (Figure 3A,B). In hIAPP-expressing cells transfected with siCHOP, thapsigargin or the combination of 25 mM glucose and palmitic acid failed to induce CHOP expression (Figure 3A,B), confirming successful knockdown of CHOP, whereas scrambled siRNA showed similar levels of CHOP when compared to treated controls. Under both conditions, the decrease in CHOP expression was associated with a decrease in the apoptotic marker cleaved caspase 3 (Figure 3C,D), suggesting that the effects observed are CHOP mediated, since the knockdown of CHOP alone is sufficient enough to prevent induced thapsigargin and high glucose and palmitic acid stress and apoptosis.

Chaperones ameliorate induced ER Stress in INS1E hIAPP cells

In order to increase endogenous chaperone expression, we used adenoviral vectors encoding for the chaperones BiP and PDI. Adenoviral BiP transduction in hIAPP-INS1E showed increased BiP protein expression with increasing MOI of adenovirus (Figure 4A). Adenoviral PDI/GFP expression was similarly tested in hIAPP-INS1E cells (Figure 4B). An MOI of 20 for each adenovirus was chosen for future experiments, based on maximal BiP or PDI/GFP expression in the absence of cell death or detectable cell toxicity (Figure 4C). Although BiP was highly expressed under basal conditions (Figure 4A) and after treatment with high glucose and palmitic acid (Figure 5A), BiP was not overexpressed in hIAPP-INS1E cells in the presence of thapsigargin (Figure 5A), suggesting that thapsigargin blunted adenoviral protein expression.

hIAPP-INS1E cells were cultured in the presence of thapsigargin-containing chemical chaperones TUDCA and PBA for 24 hours. After exposure to thapsigargin, hIAPP-INS1E cells showed high protein expression levels of ER stress markers CHOP and ATF3 (Figure 5B), confirming the mRNA expression results observed in Figure 1B and 1C. When hIAPP-INS1E cells were treated with chemical chaperones TUDCA and PBA, CHOP and

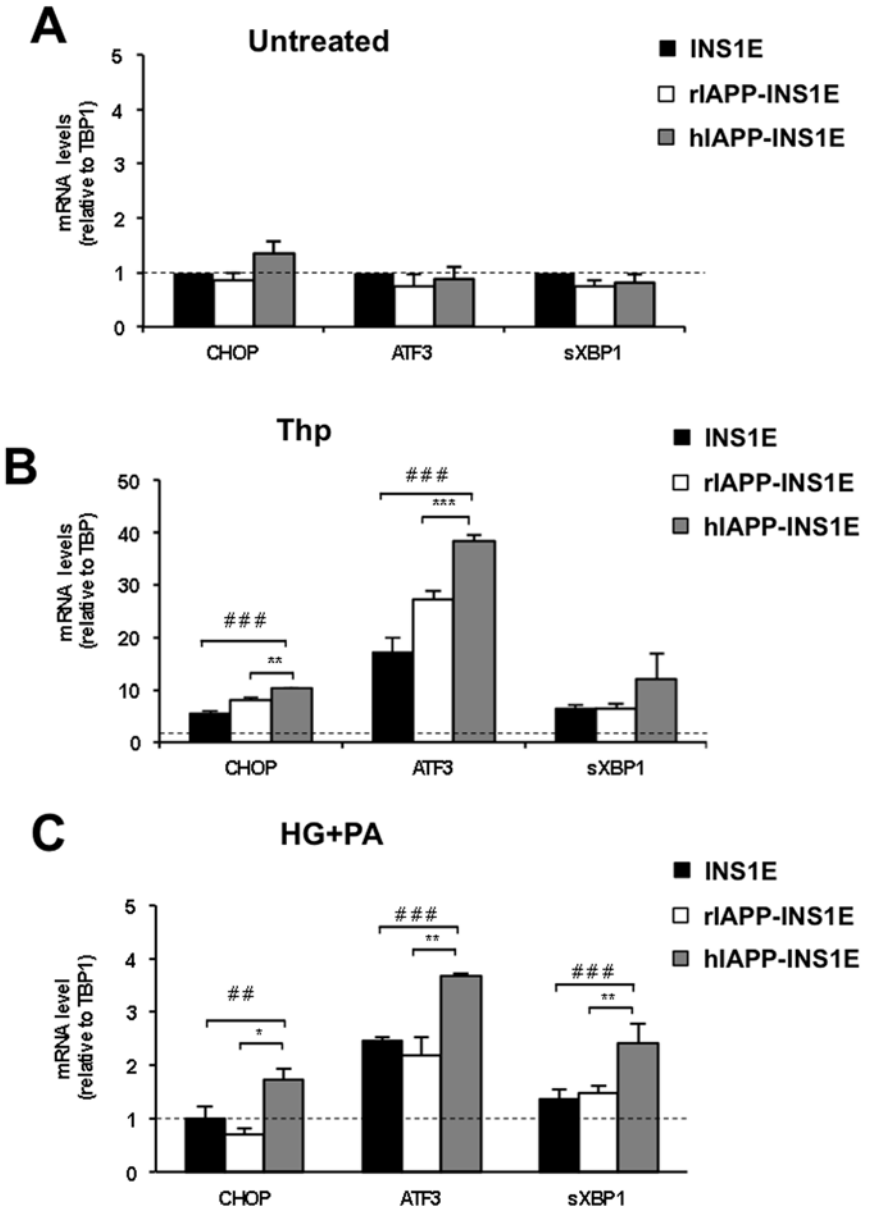

Figure 2. Thapsigargin and high glucose and palmitic acid potentiate ER stress gene expression in hIAPP-INS1E cells. ER stress expression markers CHOP, ATF3 and sXBP1 were determined by real-time PCR from hIAPP-INS1E, rIAPP-INS1E and INS1E control cells cultured at A) 11 mM glucose B) 11 mM glucose exposed to 0.5 μM of thapsigargin for 8 hours or C) 25 mM of glucose (HG) with 400 μM palmitic acid (PA) for 24 hours. Results are normalized to untreated INS1E, rIAPP-INS1E or hIAPP-INS1E cells (dashed line) and expressed as mean ± S.E.M from five independent experiments. *$p<0.05$ **$p<0.01$ and ***$p<0.001$ *vs.* rIAPP and $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ *vs.* INS1E control. n.s., not significant.
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ATF3 levels were significantly diminished (Figure 5B), although BiP levels were not recovered. Together, these results suggest that chemical chaperones TUDCA and PBA were able to reduce the hIAPP-potentiated ER stress associated with thapsigargin.

To mimic the plausible protective role of chaperones during high-glucose and palmitic acid-induced ER stress, hIAPP-INS1E cells were incubated with 25 mM glucose and palmitic acid and treated with TUDCA, PBA or previously transduced with Ad-BiP or Ad-PDI for 24 hours. hIAPP-INS1E cells exposed to high glucose and palmitic acid showed an increase in ER stress genes such as ATF3, or phospho Eukaryotic Initiation Factor 2 α (p-eIF2α), when compared to untreated controls (Figure 5C). However, hIAPP-INS1E cells treated with chaperones (TUDCA, PBA, BiP or PDI), demonstrated a decrease in ER stress by showing a decrease in ATF3 and p-eIF2α protein levels (Figure 5C). BiP levels were not affected after exposure to high glucose and palmitic acid, except when hIAPP cells were transduced with adenovirus, confirming the results observed in Figure 5A. Together these data suggest that chaperones are able to ameliorate induced-ER stress in hIAPP-expresing beta-cells; thus, improving chaperone capacity could be important in diminishing hIAPP toxicity.

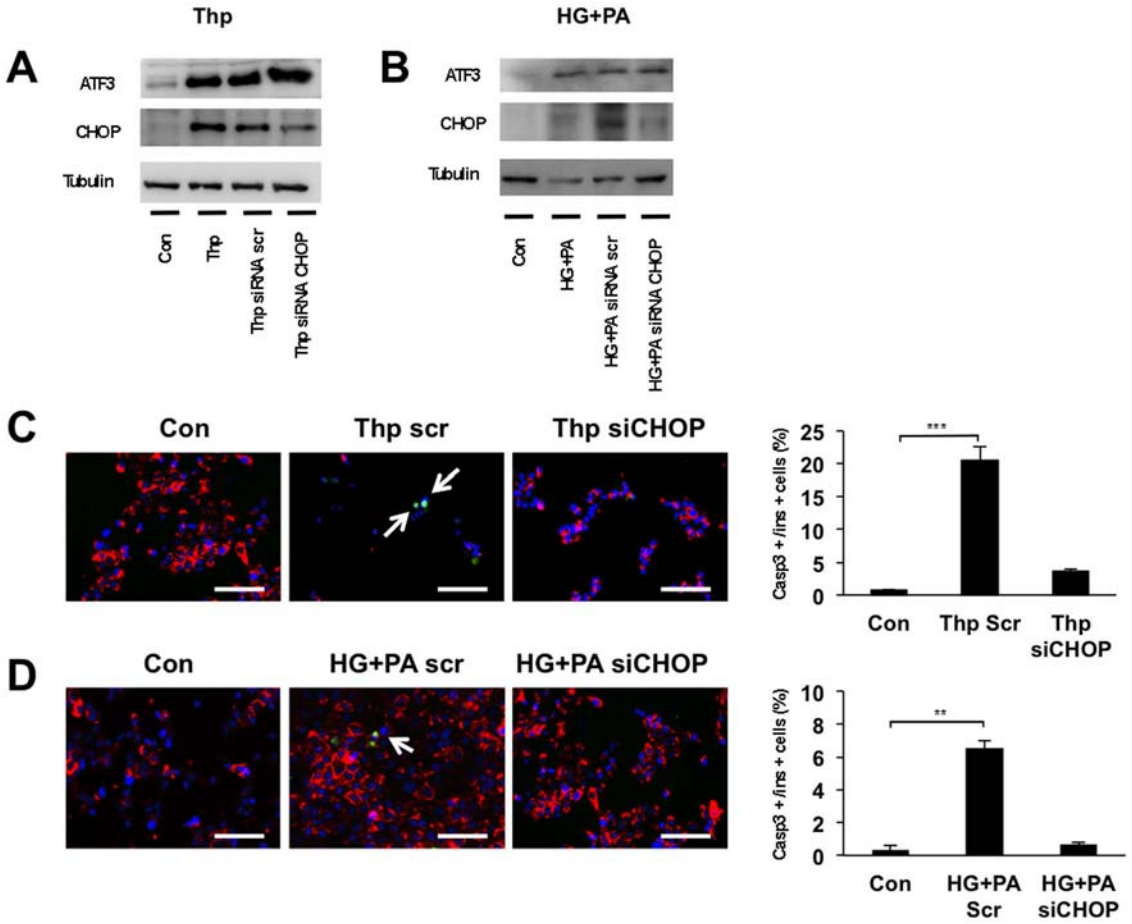

Figure 3. Knockdown of CHOP protects hIAPP-INS1E cells from induced apoptosis. hIAPP-INS1E cells were transfected with 20 μM of siRNA CHOP or siRNA scramble (siRNA scr) as a control. Twenty-four hours after transfection, cells were treated with A) 0.5 μM Thp for 8 hours or B) high glucose (25 mM) and BSA-coupled palmitic acid (400 μM; HG+PA) for 24 hours, and protein expression levels for CHOP, ATF3 and tubulin were determined. Representative Western blotting images are represented from 3 to 5 individual experiments. Immunostaining (left panel) and quantification (right panel) of insulin positive beta-cells (red), cleaved caspase 3 (green) and nuclei (blue) of hIAPP-INS1E cells (Con) and hIAPP-INS1E cells transfected with siRNA scr or siRNA CHOP previously treated with C) Thp or D) HG+PA. Note the absence of cells containing insulin and cleaved caspsase 3 staining in cells transfected with siRNA CHOP as compared to Thp or HG+PA treated controls. Scale bar is 50 μm. Quantification is normalized to number of insulin + cells and expressed as mean ± S.E.M from three independent experiments. $^{**}p<0.01$ and $^{***}p<0.001$ *vs.* controls. No statistical differences were found between controls and Thp siCHOP or HG+PA siCHOP.
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Chaperone treatment improves beta-cell function by increasing glucose-stimulated insulin secretion

To investigate the effect of chaperones on insulin secretion, hIAPP-INS1E cells were either left untreated (Con) or treated for 24 h with Ad-GFP as control, Ad-BiP, Ad-PDI and chemical chaperones TUDCA and PBA. Insulin release from untreated hIAPP-INS1E cells at 16.7 mM glucose was increased in untreated or Ad-GFP treated cells as compared to 2.8 mM glucose. However, overexpression of hIAPP alters glucose-stimulated insulin secretion when compared to INS1E control cells (Figure 6A). Nevertheless, chemical and endogenous chaperone treatment was able to restore and improve glucose-stimulated insulin secretion, although the insulin secretory response of control INS1E cells was not achieved.

The improvement in insulin secretion in hIAPP-INS1E cells treated with BiP, TUDCA and PBA does not appear to be associated with a decrease in insulin content, since all groups had similar levels of insulin in lysates before and after exposure to glucose (Figure 6B). However, PDI treatment of hIAPP-INS1E cells seems to have a detrimental effect on insulin content, suggesting that PDI may be increasing insulin secretion by degranulation of insulin vesicles (Figure 6B).

Chaperone treatment improves impaired beta-cell function as a result of high glucose and palmitic acid

In order to study the role of chaperones in insulin release under physiological stress conditions, hIAPP-expressing INS1E were exposed to high glucose and palmitic acid for 24 hours in the presence of chaperones. High glucose and palmitic acid exposure diminished glucose-stimulated insulin release from hIAPP-INS1E cells at 16.7 mM glucose when compared to untreated hIAPP-INS1E cells (Figure 7). In contrast, BiP, PDI, TUDCA and PBA treatment was able to prevent beta-cell dysfunction and maintain insulin secretory response in a similar way to untreated cells. Insulin content of hIAPP-INS1E cells stimulated at 16 mM and 2.8 mM glucose was similar in all groups (data not shown). These results demonstrate that improving chaperone capacity can ameliorate beta-cell function under stressful conditions.

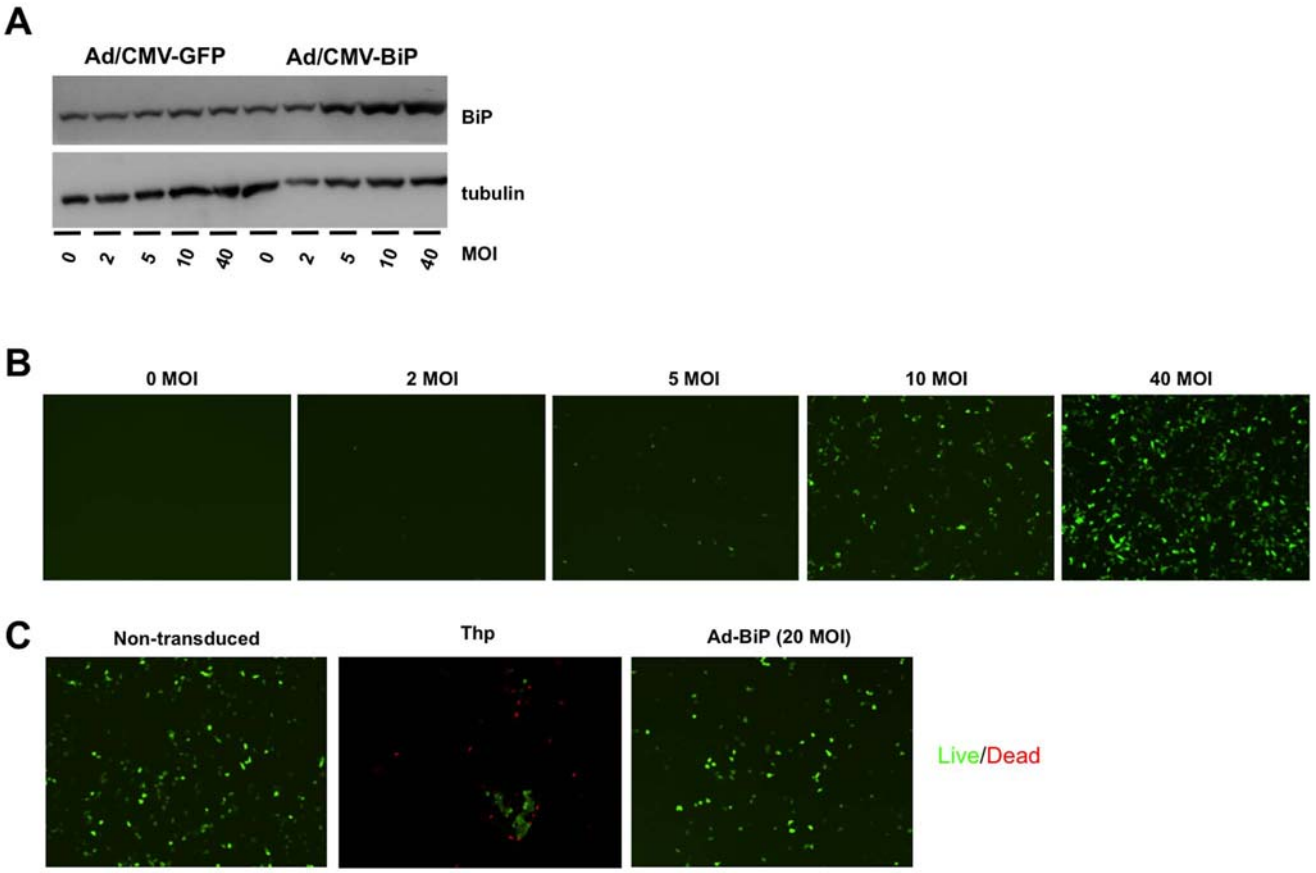

Figure 4. Increased BiP and PDI expression after adenoviral transduction does not affect cell viability. A) hIAPP-INS1E cells were transduced with different doses (MOI) for 24 hours. Representative Western blotting shows BiP protein levels at indicated doses. B) GFP expression after adenoviral (Ad-PDI/GFP) transduction in hIAPP-INS1E cells for 24 hours. Note an increase in GFP expression that correlates with an increase in MOI. C) Live/death viability assay showing no cell death in hIAPP-INS1E cells transduced for 24 hours with Ad-BiP. Western blotting and images in B and C are representative from 3 independent experiments.
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Discussion

The process of islet amyloid deposition has been recognized as a remarkable physiopathological finding involved in the failure of beta-cell function in T2D [2,3]. In the present study, we use a previously characterized rat beta-cell line overexpressing the hIAPP transgene that showed intracellular oligomers and a strong alteration to glucose-stimulated insulin and IAPP secretion [26]. We show that thapsigargin or the combination of high glucose and palmitic acid treatment potentiated ER-stress markers via CHOP pathway, and altered the secretory capacity of hIAPP-INS1E cells. By improving chaperone capacity, we have been able to recover ER stress markers and counteract the glucose-stimulated insulin secretion of hIAPP-INS1E cells.

We have previously seen that hIAPP-INS1E cells showed no change in cell death and no change in ER stress marker CHOP when compared to rIAPP-INS1E control cells [26]. Here, we found that the upstream pathways involved in ER stress, such as sXBP1 or ATF3, were not affected, confirming that hIAPP overexpression does not lead to ER stress under basal conditions (11 mM glucose). However, the role of hIAPP in ER-stress induction still needs to be elucidated. In accordance with our studies, Hull et al. demonstrated that overexpression of hIAPP transgenic mice was not associated with significant increases in the expression of ER stress markers [28]. In contrast, some reports have shown that rodent overexpression of hIAPP activates ER stress-mediated apoptosis, leading to a reduction in beta-cell mass [29]. These differences can be explained by the different levels of hIAPP expression or the use of different experimental models, such as transgenic mouse, transgenic rat or INS1E stable cell lines.

Previous studies have shown that high glucose and palmitic acid are potent inducers of ER stress [30,31]. For example, exposure of islets to high glucose concentrations induces a significant increase in apoptosis [32]. Similarly, increased concentrations of saturated fatty acids are also toxic to islets [9,33]. Saturated fatty acids impair glucose-stimulated insulin secretion and have a toxic effect on β-cells [34,35,36]. Importantly, it has been demonstrated that lipotoxicity is exacerbated in the presence of concomitantly elevated glucose levels [35]. Herein, we show that hIAPP-INS1E cells are more sensitive to exogenous ER stress inducers. Thapsigargin or high glucose and palmitic acid treatment significantly potentiated the expression of ER stress markers, such as CHOP, ATF3 or sXBP1, in hIAPP-expressing cells, when compared to rIAPP- or non-expressing controls, suggesting that hIAPP, but not rIAPP, increased sensitivity to ER stress. The potentiated ER stress activation in hIAPP-INS1E cells can be explained due to the increased aggregation capacity of hIAPP or the formation of intracellular oligomers, which may further affect ER stress [26,37,38].

We showed that thapsigargin-induced ER stress was associated with persistent protein upregulation of CHOP and ATF3 in

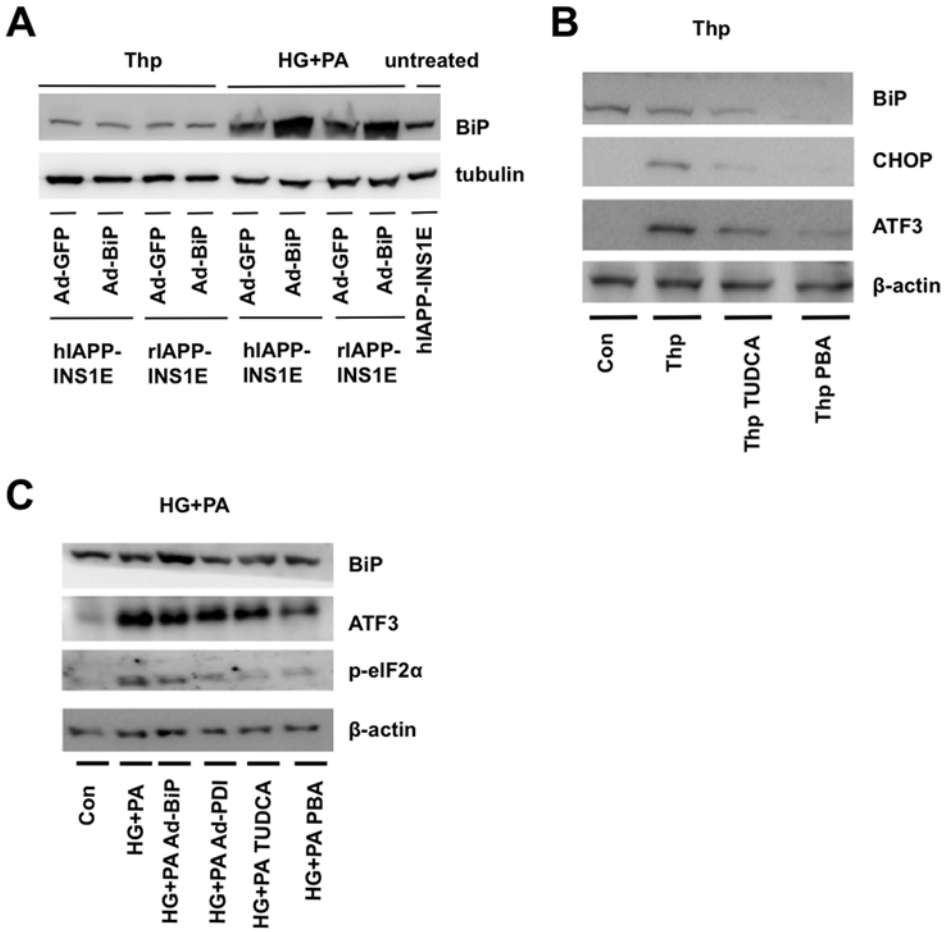

Figure 5. Endogenous and chemical chaperones decrease ER stress markers in hIAPP-INS1E cells. A) BiP and tubulin levels were determined in hIAPP-INS1E cells previously transduced with Ad-BiP or Ad-GFP for 24 hours. Representative western blotting results showing protein expression levels of CHOP, ATF3, BiP, p-eIF2α and β-actin levels in hIAPP-INS1E cells cultured with B) 0.5 μM Thp for 8 hours or C) with 25 mM of glucose and 400 μM palmitic acid (HG+PA) for 24 hours. Representative results from 3 to 5 individual experiments are shown.
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hIAPP-INS1E cells. Similarly, high glucose and palmitic acid ER stress induction was associated with p-eIF2α, ATF3 and CHOP. CHOP is a primary mediator of ER stress-induced apoptosis and is activated upon prolonged ER stress signaling. Here, we demonstrated that either thapsigargin or high glucose and palmitic acid-induced ER stress and apoptosis were dependent of CHOP. Several reports showed that treatment with saturated fatty acids cause numerous alterations that can initiate apoptosis by different mechanisms, including reactive oxygen species, mitochondrial dysfunction, generation of ceramide or induction of CHOP and caspase 3 pathway [9,13,39,40]. In accordance with our results, deletion of CHOP has been shown to enhance beta-cell function and mass in several models of beta-cell stress and T2D [41,42]. More importantly, this change preceded the induction of cleaved caspase 3, which was apparent after treatment of thapsigargin or high glucose and palmitic acid. Overall, these data suggest that CHOP plays a detrimental role in ER stress induction and that CHOP silencing may be a therapeutic approach to modulating beta-cell function and survival in T2D.

In our studies, activation of ER stress markers was reversed after treatment with chaperones. In thapsigargin or high glucose and palmitic acid treatment, the addition of chemical chaperones TUDCA and PBA was able to prevent activation of ER-stress protein markers. Similar effects were observed after transduction with adenovirus encoding for endogenous chaperones BiP and PDI after high glucose and palmitic acid treatment. In accordance with our results, BiP overexpression has been shown to diminish apoptosis by attenuating the induction of CHOP in ER stress [43].

In line with other reports, we noted that beta-cell overexpression of hIAPP shows a failure in insulin secretion in response to glucose stimulation [26,44]. The results obtained in hIAPP-INS1E cells demonstrate that treatment with chaperones BiP, TUDCA and PBA ameliorate insulin secretory response under basal conditions. In contrast, PDI showed a marked increase in insulin secretion, accompanied with a significant decrease in insulin content. Although PDI is present in human islets [45] and has been shown to play an important role in sulphide bond formation and isomeration or protein degradation [15,16,46], its overexpression has been associated with induced ER stress resulting from accumulation of proinsulin in the ER [14], suggesting that PDI overexpression may have a detrimental effect that disrupts normal insulin processing.

Treatment with high glucose and palmitic acid diminished insulin secretion in hIAPP-INS1E cells, confirming a glucolipotoxic effect [47]. Interestingly, chaperone treatment was able to recover glucose-stimulated insulin secretion. Therapeutic interventions that reduce ER stress have been studied in order to provide strategies for treating ER stress-related human diseases

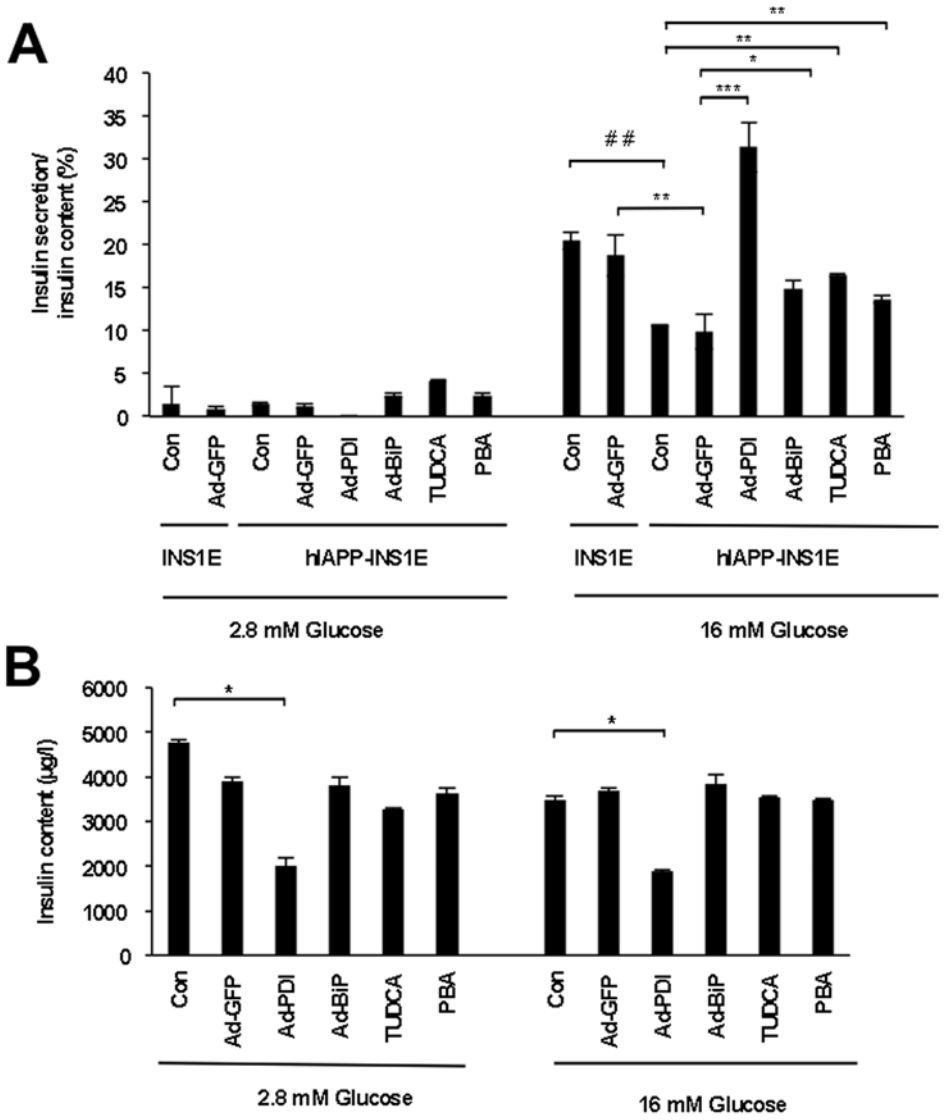

Figure 6. Chaperones ameliorate insulin secretion in hIAPP-INS1E cells. hIAPP-INS1E cells were transduced with Ad-GFP, Ad-BiP, Ad-PDI or treated with TUDCA or PBA for 24 hours. A) Glucose-stimulated insulin secretion was performed at low (2.8 mM) and high (16 mM); glucose expressed as % of insulin content. INS1E control cells were used as a control (Con). B) Total insulin content from same groups was determined in lysates. Results are expressed as mean ± S.E.M from four independent experiments. *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs. hIAPP-INS1E control, $^{\#\#\#}p<0.001$ *vs.* INS1E control. No statistical differences were found in secretion or content between Con and Ad-GFP, BiP, TUDCA and PBA.
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such as T2D [9]. BiP has been shown to be elevated in beta-cells of hIAPP-transgenic mouse models [29]. This increase of BiP can be related to the UPR in response to an increased overload of hIAPP. In addition, BiP has direct interaction with amyloidogenic peptides [48] and has been shown to attenuate the formation of amyloid-like aggregates, suppressing the misfolding of hIAPP [23]. Furthermore, transgenic mice overexpressing BiP specifically in beta-cells were protected against the injury of obesity-induced T2D, maintaining beta-cell function and improving glucose homeostasis [12]. In a similar way, BiP overexpression has been shown to improve insulin sensitivity in ob/ob mice [49]. A promising approach is the use of pharmacological agents, such as orally active chemical chaperones, which can stabilize protein conformation, improve ER folding capacity and facilitate the trafficking of mutant proteins [18,20,50,51,52,53]. Ozcan et al. have shown that chemical chaperones, such as PBA and TUDCA, reduce ER stress and restore glucose homeostasis in a mouse model of T2D [18]. In this model, the oral chemical chaperone treatment of obese diabetic mice resulted in the normalization of hyperglycemia and restoration of peripheral insulin sensitivity, thus acting as a potential antidiabetic agent [18]. Furthermore, PBA may provide health benefits by ameliorating insulin resistance and pancreatic β-cell dysfunction in obese subjects [53]. The ability of endogenous and chemical chaperones to alleviate ER stress in transgenic and obese mice models strongly supports the ER stress-based mechanistic model of T2D and demonstrates the feasibility of targeting ER function for therapeutic goals.

In conclusion, our study suggests that ER stress plays a causal role in beta-cell dysfunction in a context of hIAPP overexpression. Furthermore, our results suggest that ameliorating chaperone capacity can be of potential interest for preserving beta-cell function in T2D.

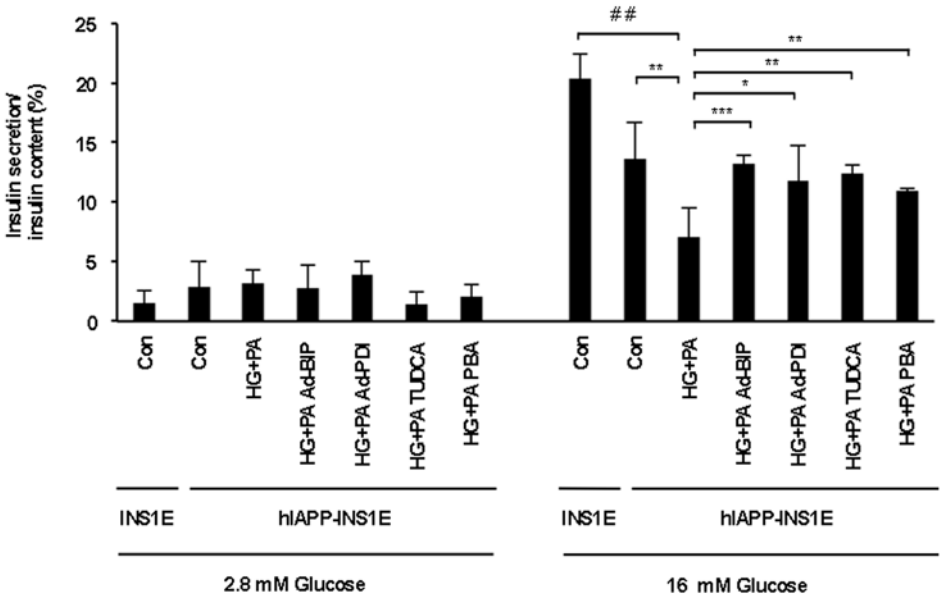

Figure 7. Chaperone treatment prevents beta-cell dysfunction under high glucose and palmitic acid treatment. hIAPP-INS1E cells were transduced with Ad-BiP, Ad-PDI or treated with TUDCA or PBA for 24 hours. After 24 hours, cells were treated with 25 mM of glucose and palmitic acid (HG+PA). Glucose-stimulated insulin secretion was performed at low (2.8 mM) and high (16 mM) glucose using INS1E cells as control as expressed by % of insulin content. Insulin levels were determined by ELISA. Results are expressed as mean ± S.E.M from three independent experiments. $^{\#\#}p<0.05$ *vs* INS1E control, $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ *vs*. hIAPP-INS1E cells treated with HG+PA. No statistical differences were found between Con and BiP, PDI, TUDCA and PBA.
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Author Contributions

Conceived and designed the experiments: LC JM JMS AN. Performed the experiments: LC JM GAV MV LVF. Analyzed the data: LC JM LVF. Contributed reagents/materials/analysis tools: LC JM AN. Contributed to the writing of the manuscript: LC JM JMS AN.

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REVIEW

Stress and the inflammatory process: a major cause of pancreatic cell death in type 2 diabetes

Joel Montane
Lisa Cadavez
Anna Novials

Diabetes and Obesity Research Laboratory, Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS), Hospital Clínic de Barcelona, Barcelona, Spain; Centro de Investigación Biomédica en Red de Diabetes y Enfermedades Metabólicas Asociadas (CIBERDEM), Barcelona, Spain

Abstract: Type 2 diabetes (T2D) is a complex metabolic disorder characterized by hyperglycemia in the context of insulin resistance, which precedes insulin deficiency as a result of β-cell failure. Accumulating evidence indicates that β-cell loss in T2D results as a response to the combination of oxidative stress and endoplasmic reticulum (ER) stress. Failure of the ER's adaptive capacity and further activation of the unfolded protein response may trigger macroautophagy (hereafter referred as autophagy) as a process of self-protection and inflammation. Many studies have shown that inflammation plays a very important role in the pathogenesis of T2D. Inflammatory mechanisms and cytokine production activated by stress via the inflammasome may further alter the normal structure of β-cells by inducing pancreatic islet cell apoptosis. Thus, the combination of oxidative and ER stress, together with autophagy insufficiency and inflammation, may contribute to β-cell death or dysfunction in T2D. Therapeutic approaches aimed at ameliorating stress and inflammation may therefore prove to be promising targets for the development of new diabetes treatment methods. Here, we discuss different mechanisms involved in stress and inflammation, and the role of antioxidants, endogenous and chemical chaperones, and autophagic pathways, which may shift the tendency from ER stress and apoptosis toward cell survival. Strategies targeting cell survival can be essential for relieving ER stress and reestablishing homeostasis, which may diminish inflammation and prevent pancreatic β-cell death associated with T2D.

Keywords: endoplasmic reticulum stress, chaperones, autophagy, inflammation, apoptosis, unfolded protein response

Introduction

Type 2 diabetes (T2D) is characterized by hyperglycemia in the context of insulin resistance and β-cell dysfunction.[1] Over time, islet β-cell function compensates for the insulin resistance existing in peripheral tissues, resulting in defects in insulin secretion that impair the regulation of blood glucose levels.[1–3] Moreover, postmortem studies on β-cell loss in T2D have concluded that there is a marked reduction in β-cell mass,[4–6] which is probably due to an increase in apoptosis rather than a decrease in β-cell replication. In addition to the increased β-cell workload in response to the abnormally high demand induced by insulin resistance, several factors likely play a role in this process. For example, high levels of glucose and saturated fatty acids in the blood, increased expression of islet amyloid polypeptide (IAPP), which is mainly responsible for amyloid deposits in the pancreas,[7,8] as well as inflammatory cytokines released from visceral adipose tissue,[9] may be involved as inductors of oxidative stress and endoplasmic reticulum (ER) stress.

Correspondence: Anna Novials
Diabetes and Obesity Research Laboratory, Institut d'Investigacions Biomèdiques August Pi I Sunyer (IDIBAPS), Hospital Clínic de Barcelona, c/Rosello, 149-153 08036, Barcelona, Spain
Tel +34 93 227 5400
Fax +34 93 312 9403
Email anovials@clinic.ub.es

These factors, together with the activation of the local inflammatory response signal the pathways leading to β-cell exhaustion and death.

A growing number of studies implicate ER stress in the loss and death of β-cells during the evolution of T2D.[10,11] The ER is considered a vital organelle for protein synthesis and maturation, quality control, and secretion;[12,13] however, these processes require a stable environment for balancing ER protein load and ER folding capacity. A variety of factors can disturb the proper functioning of the ER, leading to ER stress and inflammation as well as the induced synthesis of pro-inflammatory cytokines, including tumor necrosis factor-α and interleukin (IL)-6, via inflammasome activation.[11] In addition, the unfolded protein response (UPR) activates other pathways, such as oxidative stress and autophagy[10,14,15] which eventually lead to cell death or cell survival, depending on the balance of such factors in the cellular milieu.

In this review, we address the central mechanisms underlying ER stress, oxidative stress, autophagy, and inflammation, as well as the pathways that contribute to pancreatic β-cell death in the framework of T2D.

The link between stress and inflammation in pancreatic β-cells

ER stress and the UPR response

Providing a high-fidelity quality control system, the ER has developed an elaborate adaptive response known as the UPR, in which there is a perfect recognition of misfolded proteins and an efficient removal of these proteins from the ER lumen in order to protect and alleviate cells from ER stress. The UPR attempts to reestablish homeostasis and restore ER function by diminishing protein translation and activating a series of mechanisms that increase the biosynthetic capacity of the secretory pathway, such as ER chaperones. For this, a complex signaling network is initiated by three ER transmembrane kinases: protein kinase R-like endoplasmic reticulum kinase (PERK); inositol-requiring enzyme 1 (IRE1); and activating transcription factor (ATF)6 (Figure 1). Chaperone 78 kDa glucose-regulated protein (GRP78), also referred to as BiP (immunoglobulin heavy chain binding protein), is a central regulator of ER stress due to its controlling of the activation of transmembrane ER stress sensors (PERK, IRE1, and ATF6) through a binding-release mechanism.[11]

A reduction in protein translation and in ER workload are the first responses to counteract ER stress. This is mediated by PERK, which phosphorylates the α subunit of eukaryotic translation initiation factor 2, reducing global protein synthesis

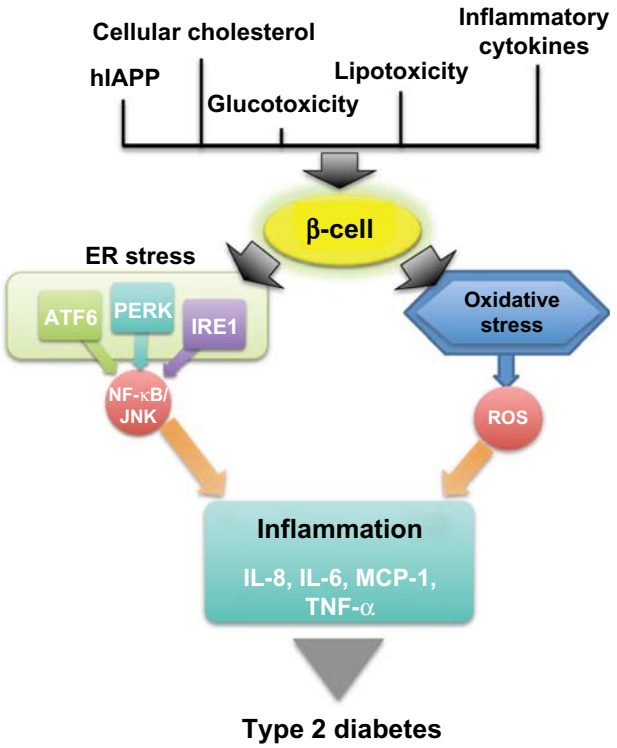

Figure 1 The link between β-cell stress and inflammation in type 2 diabetes.
Notes: Cellular cholesterol, human islet amyloid polypeptide, glucotoxicity, lipotoxicity or inflammatory cytokines induce cellular stress through activation of ER stress or oxidative stress. In response to ER stress, the three branches of the unfolded protein response are activated. PERK, ATF6, and IRE1 result in activation of JNKs and/or NF-κB, switching on expression of a variety of different genes involved in inflammatory pathways, such as cytokines, chemokines, or ILs. On the other hand, oxidative stress induces an excess production of ROS, which activates several inflammatory signaling cascades that will contribute to inflammation. Expression of proinflammatory molecules might attract local inflammatory cells, which may further exacerbate the local inflammation, causing β-cell apoptosis and type 2 diabetes.
Abbreviations: hIAPP, human islet amyloid polypeptide; ER, endoplasmic reticulum; ATF, activating transcription factor; PERK, protein kinase R-like endoplasmic reticulum kinase; IRE1, inositol-requiring enzyme 1; NF-κB, nuclear factor κ-light-chain-enhancer of activated B cells; JNK, c-Jun N-terminal kinases; ROS, reactive oxygen species; IL, interleukin; MCP, monocyte chemotactic protein; TNF, tumor necrosis factor.

and inducing the translation of ATF4 messenger (m)RNA. This transcription factor activates the translation of ATF3 and CCAAT/enhancer-binding protein (C/EBP) homologous protein (CHOP). The loss of PERK expression in humans and mice has been associated with a failure to properly regulate the UPR response, underlying dysfunction in the ER stress and UPR pathways, which can result in increased cell death and diabetes.[16] Although PERK expression in adult β-cells does not appear to be required for maintaining β-cell function, mutations in PERK result in the elevation of ER stress markers, leading to a form of permanent neonatal diabetes in humans,[17–19] suggesting that PERK may play an important role in controlling ER stress during fetal development.

IRE1, the second pathway of the UPR, is highly expressed in the pancreas and is considered a central regulator of ER

stress signaling, playing a crucial function in the regulation of protein biosynthesis.[20] A previous study has shown that IRE1 signaling knockdown in vitro decreases insulin biosynthesis at the translation and protein-folding level.[21] Once activated, IRE1 cleaves the mRNA encoding X-box binding protein 1 (XBP1), leading to an activated version of the transcription factor spliced XBP1. Once translocated to the nucleus, the spliced XBP1 protein initiates several transcriptional programs that upregulate ER expansion and biogenesis, increase protein entry into the ER for maturation, and degrade misfolded proteins.[22] Lee et al demonstrated that β-cell-specific XBP1-deficient mice elicited an impairment in β-cell proliferation, proinsulin processing, and insulin secretion, along with a hyperactivation of IRE1,[23] suggesting that XBP1 is critical in achieving optimal insulin secretion and glucose control and thus may be considered a key regulator of the UPR.

The third pathway of the UPR initiates with the activation of the basic leucine zipper domain protein ATF6. ATF6 activation stimulates its own translocation to the Golgi, where site-1 protease and site-2 proteases are cleaved and conducted to the nucleus to target transcription chaperones, elements of the ER-associated degradation pathway, and the upregulation of XBP1. Under chronic ER stress, ATF6 attempts to suppress the apoptotic UPR signaling cascade by upregulation of the PERK and IRE1 pathways. Recent reports have tried to elucidate the role of ATF6 in β-cell function. No association has been found between ATF6 polymorphisms in the general population and cohorts of type 2 diabetic patients;[24] however, ATF6 knockdown in insulinoma cells showed a decrease in ER chaperones and induced cell apoptosis without any changes in the PERK and IRE1 pathways.[25] Similarly, ER stress-induced activation of ATF6 has been shown to suppress insulin gene expression,[26] suggesting that ATF6 plays an important role in β-cell dysfunction.

ER stress and the inflammatory signal

The three branches of UPR response can trigger inflammatory signals through different branches that converge in signaling pathways involving c-Jun N-terminal kinases (JNKs) and the nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) (Figure 1). The activation of these kinases highlights the overlap of metabolic and immune pathways, since these are the same kinases that are activated by innate immune responses.[27,28] JNKs are considered to play an important role in ER stress in mouse models of diabetes. For instance, an increase in JNK activity promotes insulin resistance in peripheral tissues and in pancreatic β-cells without affecting cell viability.[29,30] The importance of the JNK pathway in stress has also been observed in knockout mice, in which suppression of the JNK pathway protects β-cells against oxidative stress induction.[31]

The pathway of the UPR involving IRE1 can, by different mechanisms, trigger an inflammatory signaling pathway through the activation of JNKs.[32] In addition, through multiple mechanisms, both the IRE1 and PERK pathways can also lead to the activation of the NF-κB pathway,[11] which also plays a critical role in the induction of multiple inflammatory mediators and has been implicated in insulin resistance.[11,33] ATF6 has also been linked to inflammatory signaling. Genetic and pharmacological inhibition of ATF6 significantly suppresses NF-κB activation, which can transcriptionally regulate many other inflammatory genes.[34] Activation of either JNKs or NF-κB pathways in pancreatic β-cells has been reported to cause increased expression of proinflammatory molecules, such as IL-8, IL-6, monocyte chemotactic protein-1, and tumor necrosis factor-α,[35] that have a detrimental effect on cell survival and function.[36–38] Local chemokine and cytokine release can also contribute to the inflammatory milieu, attracting host macrophages to the pancreatic β-cells, which further propagate local inflammation.[39,40] In addition, the NF-κB pathway has been shown to activate the NLRP3 inflammasome, a multi-protein, cytosolic molecular platform that controls the activation of caspase 1, and the secretion of proinflammatory cytokines interleukin IL-1β and IL-18 in metabolic stress.[41,42] Inflammation induced by inflammasome-dependent proinflammatory cytokines may produce insulin resistance or cause the death of pancreatic β-cells, leading to development of diabetes (Figure 1).[42]

Oxidative stress and the inflammatory signal

In T2D, when insulin demand is constantly elevated, reactive oxygen species (ROS) generation is increased by mitochondrial respiration, which saturates the neutralizing capacity of antioxidants, resulting in oxidative stress. β-cells are more susceptible to oxidative stress when compared to other cell types, probably due to their low antioxidant capacity. β-cells have low levels of antioxidant enzyme expressions, such as catalase and glutathione peroxidase, making β-cells more vulnerable to free radical damage when exposed to oxidative stress.[43] In addition, β-cells display highly efficient glucose uptake when exposed to high glucose concentrations, due to the expression of glucose transporter 2. Thus, it is likely that oxidative stress plays a major role in β-cell dysfunction in T2D. Exposure of rat islets to high concentrations of glucose

resulted in an increased production of intracellular ROS.[44] Furthermore, when human islets were incubated with high glucose concentrations, the levels of intracellular peroxide were increased within the islets.[45] Similarly, elevated markers of oxidative stress have been found in the plasma and urine of type 2 diabetic patients, as well as decreased levels of antioxidant molecule glutathione in their blood cells.[46] In β-cells, elevated ROS levels lead to impaired insulin secretion and contribute to insulin resistance in T2D.[47,48] Under normal conditions, ROS are finely regulated to avoid oxidative damage to cellular processes.[49] Prolonged ER stress can also accumulate ROS through a PERK-mediated pathway, promoting a state of oxidative stress.[50] To protect itself from the highly toxic radicals, the β-cell must metabolize ROS by using cellular antioxidants, including glutathione peroxidase, catalase, thioredoxin, and superoxide dismutase, among others.[51] In addition, glutathione, the primary intracellular antioxidant, has been reported to be low in diabetic patients.[52,53] After treatment with sulfonylurea, a reduction in oxidative stress was observed, with patients showing a decrease in lipid peroxidation and an increase in glutathione circulating levels, with levels almost reaching those found in a nondiabetic control group.[53]

Inflammation resulting from oxidative stress is a key component for many human diseases, such as metabolic syndrome or T2D.[27] ROS production is capable of acting as a signaling molecule, but also inflicts oxidative damage by oxidizing fatty acids, DNA, RNA, amino acids, and cofactors.[54] ROS have been shown to play an important role in various cellular processes, including differentiation, autophagy, metabolic adaptation, and inflammation.[55] High ROS levels activate several inflammatory signaling cascades, leading to the transcription of the NF-κB pathway, monocyte chemotactic protein-1, cellular adhesion molecules, nitric oxide, transforming growth factor-β, connective tissue growth factor, and ILs.[27,56,57] Consequently, expression of proinflammatory molecules might attract inflammatory cells such as macrophages to the site and further exacerbate the local inflammation. ROS production in adipocytes can also lead to an increased production of proinflammatory cytokines that can affect β-cells in a paracrine manner.[58]

Stress, inflammation, and the activation of apoptotic signaling

Linkers of stress and apoptosis

Metabolically stressed human β-cells display markers of ER stress and activation of inflammation and apoptosis pathways.[10,11,59–62] ER stress and oxidative stress are intricately related and represent possible mediators that link toxic stimuli with target molecules in the apoptotic cascade. As far as we know, the apoptotic pathways that may be activated by ER stress are also activated by oxidative stress and inflammatory signals (Figure 1). At least three parallel pathways are involved in the stress-mediated apoptosis: activation of CHOP; activation of the IRE1–JNK pathway; and activation of caspase 12 (Figure 1).[63,64]

CHOP signaling is activated in β-cells under conditions of metabolic stress, and the deletion of CHOP has been demonstrated to enhance β-cell function and mass in several mouse models of diabetes.[65] In this regard, islets from CHOP knockout mice have fewer apoptotic cells and show an increased expression of UPR genes. Furthermore, CHOP deletion delays the onset and severity of the diabetic phenotype.[65,66] Additional mechanisms of apoptotic induction have been associated with particular branches of the ER stress pathway. ATF4 can promote apoptosis by suppression of B-cell lymphoma 2.[67] IRE1 promotes JNK signaling through a mitogen-activated protein kinase 1 pathway.[67,68] IRE1 also promotes the activation of caspase-12. Procaspase-12 is localized to ER membranes and undergoes cleavage during ER stress in murine cells, promoting the downstream cleaving of caspase-3, the last effector caspase of the apoptotic cascade.[68,69] Pharmacologically induced ER stress has given additional insight into mechanisms by which ER stress may promote apoptosis.[69] Such agents cause mitochondrial cytochrome-c release and loss of mitochondrial transmembrane potential, causing ER stress-induced apoptosis. Moreover, perturbed ER Ca^{2+} homeostasis may contribute to apoptosis following induction of ER stress,[69] since knockout mice lacking the ER Ca^{2+} channel Wsf1 are particularly susceptible to ER stress-induced apoptosis.[70] Overall, when ER stress-induced apoptosis causes the loss of a large number of β-cells, insulin secretory capacity is impaired, resulting in T2D (Figure 1).

Triggers of stress, inflammation, and apoptosis

Multiple physiological and pathological conditions, including the accumulation of misfolded proteins, such as insulin or human IAPP (hIAPP), are responsible for the loss of ER homeostasis in β-cells (Figure 1).[11,71] In some cases, protein overexpression in cells of transgenic mice can trigger ER stress and apoptosis due to a high biosynthetic misfolded load.[60,72,73] For example, studies in the Akita mouse have shown that ER stress, secondary to the misfolding of mutated insulin, leads to β-cell death and glucose intolerance.[73] The loss of β-cell mass in diabetes is exacerbated by islet amyloid

deposits that correlate with the severity of the disease in humans. β-cell apoptosis is also observed in human pancreatic sections and postmortem islet grafts in correlation with amyloid deposition levels.[71,74,75] Oligomers of human IAPP have been shown to increase inflammation in β-cells via the inflammasome.[76] Comparably, hIAPP can form proinflammatory oligomers and fibrils that contribute to islet inflammation by recruiting and activating macrophages in vivo.[39,76] Nevertheless, the role of hIAPP and ER stress still needs to be elucidated. Some reports show that ER stress-mediated apoptosis is exacerbated in rodent cells expressing amyloidogenic isoforms of hIAPP in β-cells, leading to a reduction of β-cell mass in hIAPP transgenic mice and rats.[61,71] In addition, Casas et al demonstrated that extracellular hIAPP aggregation is associated with ER stress responses in mouse β-cells, by an intracellular signaling that involves downstream inhibition of the ubiquitin–proteasome pathway, contributing to β-cell apoptosis.[77,78] Nevertheless, in a rat pancreatic β-cell line overexpressing hIAPP, the detection of toxic intracellular oligomers, which lead to defective insulin and IAPP secretion levels in response to glucose, did not change the expression of genes involved in ER stress.[79] These results agree with other findings with hIAPP transgenic mice, in which the authors demonstrated that amyloid formation was not associated with significant increases in the expression of ER stress markers.[80] The discrepancy in these results may be explained by differences in the ratio of IAPP and insulin produced by the different models used, ranging from low to significantly high levels of IAPP.

The synergistic toxic effect of hyperglycemia and hyperlipidemia is now well recognized as a contributor toward β-cell death.[81] Exposure of islets to high glucose concentrations induces a significant increase in apoptosis.[81] Similarly, high glucose concentrations increased IL-1β, followed by NF-κB activation from nondiabetic islet donors.[82] Increased concentrations of saturated fatty acids are also toxic to islets. Saturated fatty acids impair insulin gene expression and glucose-induced insulin secretion.[83–85] Palmitate induces ER stress via NF-κB activation, contributing to islet inflammation.[86] In addition, fatty acids can have a toxic effect on β-cells, since treatment of human or mouse islets with palmitate induces apoptosis.[87] Importantly, it has been demonstrated that lipotoxicity only occurs in the presence of concomitantly elevated glucose levels.[84] Other mediators include islet cholesterol accumulation as an important cause of lipotoxic stress in β-cells. Cellular cholesterol homeostasis is important for normal β-cell function. Thus, disruption of cholesterol transport by decreased function of the Adenosine triphosphate (ATP)-binding cassette (ABC) transporter Adenosine triphosphate-binding cassette transporter 1 (ABCA1), a cholesterol efflux regulatory protein responsible for cholesterol transport in β-cells, results in impaired insulin secretion.[88–90] In addition, the combined deficiency of ABCA1 and ABCG1 also results in significant islet inflammation, as indicated by the increased expression of IL-1β and macrophage infiltration.[91]

The role of stress and autophagy in apoptosis induction

The ER stress pathway is directly involved in the induction of autophagy. Autophagy is a degradation pathway responsible for the large turnover of intracellular proteins and organelles via lysosomal degradation.[92] Autophagy is a highly regulated process that can either be involved in the turnover of long-lived proteins and whole organelles in a generalized fashion, or specifically target distinct organelles.[92] Thus, autophagy, together with apoptosis, is a process through which damaged or aged cells or organelles can be eliminated.[92] Autophagy may be either a mechanism to avoid apoptosis (by eliminating old or damaged organelles) or a mechanism to induce apoptosis by autophagic-induced cell death.[93] Autophagy and apoptosis may be triggered by common upstream signals, resulting in combined autophagy and apoptosis. For example, autophagy can destroy large proportions of the cytosol or organelles, causing irreversible cellular collapse.[93] Autophagy can also be a response to stress stimuli by triggering apoptosis or necrotic cell death. Likewise, several signal transduction pathways related to cellular stress (such as oxidative or ER stress pathways) can elicit both autophagy and apoptosis.[93] Autophagy can also be induced by proteasome inhibition under conditions of ER stress, demonstrating that autophagy and apoptosis share many common inducers.[93]

Several reports have studied the role of autophagy in pancreatic β-cells. Diabetic db/db or nondiabetic C57Bl/6 mice fed with a high-fat diet have shown upregulated autophagosome formation in β-cells.[94,95] Furthermore, genetic ablation of Autophagy-related protein 7 (ATG7) (an essential gene for autophagosome formation) in β-cells was shown to have resulted in the degeneration of islets and impaired glucose tolerance with reduced insulin secretion.[94,96] These findings were associated with an increased level of apoptosis and a decreased proliferation, which contributed to the loss of β-cell mass. It has been described that increased expression of hIAPP in transgenic mice and rats leads to an impaired autophagy pathway, due to the disruption of

lysosome-dependent degradation.[97] In addition, inhibition of lysosomal degradation increases the vulnerability of β-cells to hIAPP-induced toxicity and, conversely, stimulation of autophagy protects β-cells from hIAPP-induced apoptosis.[97] In humans, the autophagy pathway also declines with age and is impaired in β-cells in type 2 diabetic patients.[98,99] These studies suggest that autophagy is necessary to maintain the structure, mass, and function of pancreatic β-cells, and its impairment may participate in the mechanisms that cause β-cell failure and T2D.

Moving the balance toward homeostasis and promoting β-cell survival

Pancreatic β-cells need to increase protein synthesis during acute or chronic stimulation. This causes a burden on the ER that may activate autophagy and the UPR response, which may lead to pancreatic cell death. Together, cell survival and cell death factors represent key opposing forces underlying stress response (Figure 2). As previously seen, many factors can mediate the respective outcome of this antagonistic process. If a prolonged imbalance persists, the response system initiates proapoptotic mechanisms that eventually will lead to pancreatic cell death and dysfunction associated with T2D (Figure 2). Thus, therapeutic interventions that target molecules of the UPR component or reduce ER stress, such as increasing cellular antioxidants, chaperone capacity, or autophagy levels, may bring the balance toward homeostasis and provide promising strategies for treating ER stress-related human diseases such as T2D (Figure 2).

The inhibition of intracellular free radical formation may represent one therapeutic strategy for preventing oxidative stress. Antioxidants act at different levels, inhibiting the formation of ROS, scavenging free radicals or increasing their own defense enzyme capabilities. Therapeutic measures designed to increase intrinsic antioxidant activity within the islet may also protect it against the oxidative stress associated with glucose toxicity (Figure 2). The transduction of islets with adenovirus encoding for the antioxidant enzyme, glutathione peroxidase, has been shown to protect islets against the intra-islet peroxide levels produced by high glucose concentrations.[100] Furthermore, exogenous antioxidants can compensate for the lower plasma antioxidant levels often observed in T2D. Antioxidants such as N-acetylcysteine, vitamin C, and α-lipoic acid are effective in reducing diabetic complications,[101,102] indicating that antioxidants may prove an essential tool in the investigation of oxidative stress-related diabetic pathologies.

An increasing body of evidence suggests that chaperones exert important protective effects in the decrease of ER stress, protein aggregation, and the pathophysiology of amyloid deposition. Overexpression of certain particular ER chaperones in cell systems can protect cells against cell death caused by disturbances of ER homeostasis.[103–106] Of interest, transgenic mice overexpressing the molecular chaperone

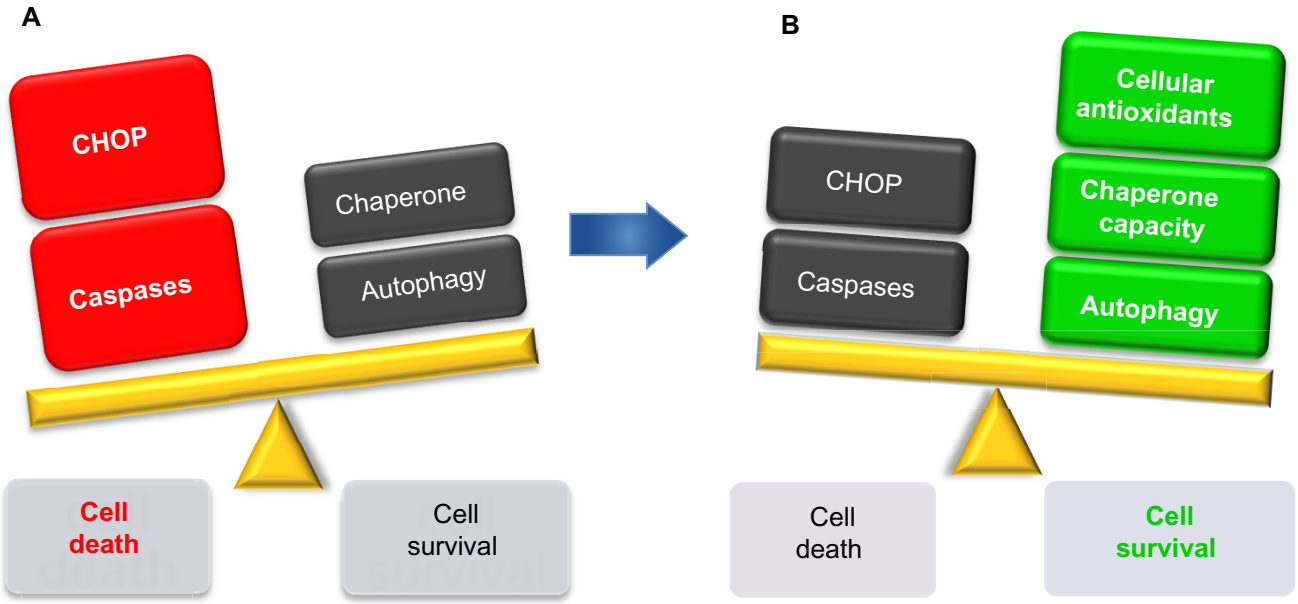

Figure 2 Moving the balance toward prosurvival strategies.
Notes: As a result of chronic stress and inflammation, β-cells may undergo cell death through activation of C/EBP homologous protein (CHOP) and caspases (**A**). Adaptive responses to acute stress, such as an increase in chaperone capacity, an increase of cellular antioxidants, or an improved autophagy pathway, may move the balance toward cell survival, leading the β-cell to homeostasis (**B**).
Abbreviation: C/EBP, CCAAT/enhancer-binding protein.

GRP78/BiP specifically in β-cells are protected against the injury of obesity-induced T2D, maintaining β-cell function and improving glucose homeostasis.[104] Overexpression of BiP attenuates fatty acid-induced ER stress and apoptosis in hepatocytes.[106] Furthermore, BiP is one of the chaperones responsible for trafficking hIAPP through the ER and Golgi in human β-cells.[107] Efforts to understand the impact of chaperones may provide insights into the formation of misfolded hIAPP, which, consequently, might be a speculative approach for preventing amyloid formation, which may lead to inflammation and β-cell apoptosis in T2D (Figure 2). Few investigations have been performed on inhibiting the aggregation of IAPP. The small interfering RNA-mediated suppression of human amyloid polypeptide expression inhibits islet amyloid formation and enhances the survival of human islets in culture.[108] Similarly, peptide-based amyloid inhibitors have been seen to enhance the survival of cultured human islets.[109] Thus, inhibitors of IAPP synthesis or aggregation may have therapeutic value in diminishing amyloid formation in T2D.

Recent reports suggest that pharmacological agents can directly activate or deactivate UPR components and can potentially be useful in treating T2D. A promising approach is the use of pharmacological agents, such as orally active chemical chaperones, which can stabilize protein conformation, improve ER folding capacity, and facilitate the trafficking of mutant proteins.[110–113] Ozcan et al have shown that chemical chaperones, such as 4-phenyl butyric acid and taurine-conjugated ursodeoxycholic acid, reduce ER stress and restore glucose homeostasis in a mouse model of T2D.[114] In this model, the oral chemical chaperone treatment of obese diabetic mice resulted in the normalization of hyperglycemia and restoration of peripheral insulin sensitivity, thus acting as a potential antidiabetic agent.[114] Chemical chaperones have also been tested in obese human subjects;[115,116] 4-phenyl butyric acid, for instance, may provide health benefits by ameliorating insulin resistance and pancreatic β-cell dysfunction in obese subjects.[115] The ability of endogenous and chemical chaperones to alleviate ER stress in transgenic and obese mice models strongly supports the ER stress-based mechanistic model of T2D and demonstrates the feasibility of targeting ER function for therapeutic goals.

Several studies have focused on the prosurvival role of autophagy. As previously discussed, autophagy is activated in response to ER stress and helps cellular adaptation to stress, via clearance of misfolded proteins.[117] Thus, stimulation of autophagy may improve ER stress in diabetes. Treatment with rapamycin, an autophagy inducer, used in diabetic Akita mice, improved diabetes, increased pancreatic insulin content, and prevented β-cell apoptosis.[118] In contrast, the same study showed that inhibition of autophagy exacerbated stress and abolished the anti-ER stress effects of rapamycin.[118] In a similar manner, increasing autophagy by overexpression of scaffold protein p62, which delivers polyubiquitinated proteins to autophagy, confers a protective role against hIAPP-induced apoptosis by sequestrating protein targets for degradation.[97] Such evidence highlights an important role for autophagy in protection against toxic oligomer-induced apoptosis in β-cells. Thus, strategies that target ER stress in β-cells will promote β-cell survival and function in T2D.

Summary

A great variety of stimuli, such as IAPP, cytokines, cellular cholesterol, or high glucose and lipids levels in the blood, can disturb ER homeostasis, leading to oxidative and ER stress, inflammation, and pancreatic β-cell death. Elucidating the cellular mechanisms of stress, inflammation, and cell death has contributed toward our understanding of these processes, which may lead to new therapeutic agents for treating T2D. Strategies targeting the balance toward prosurvival have proven to be essential for the shift from stress to homeostasis, which may prevent pancreatic β-cell death associated with T2D.

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Disclosure

The authors report no conflicts of interest in this work.

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