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The Role of Endoplasmic Reticulum Stress Signaling in Pancreatic Beta Cells: a Dissertation

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THE ROLE OF ENDOPLASMIC RETICULUM STRESS SIGNALING IN PANCREATIC BETA CELLS

A Dissertation Presented

By

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"Appreciation is a wonderful thing. It makes what is excellent in others belong to us as well." –Voltaire

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ABSTRACT

Protein folding in the endoplasmic reticulum (ER) is essential for proper cellular function. However, the sensitive environment in the ER can be perturbed by both pathological processes as well as by physiological processes such as a large biosynthetic load placed on the ER. ER stress is a specific type of intracellular stress caused by the accumulation of immature or abnormal misfolded or unfolded proteins in the ER. Simply defined, ER stress is a disequilibrium between ER load and folding capacity. Cells have an adaptive response that counteracts ER stress called the "Unfolded Protein Response" (UPR). The ability to adapt to physiological levels of ER stress is especially important for maintaining ER homeostasis in secretory cells. This also holds true for pancreatic β-cells, which must fold and process large amounts of the hormone insulin.

Pancreatic β-cells minimize abnormal levels of glycemia through adaptive changes in the production and regulated secretion of insulin. This process is highly sensitive, so that small degrees of hypo- or hyperglycemia result in altered insulin release. The frequent fluctuation of blood glucose levels in humans requires that β-cells control proinsulin folding in the ER with exquisite sensitivity. Any imbalance between the load of insulin translation into the ER and the actual capacity of the ER to properly fold and process the insulin negatively affects the homeostasis of β-cells and causes ER stress.

In this dissertation, we show that Inositol Requiring 1 (IRE1), an ER-resident kinase/endoribonuclease and a central regulator of ER stress signaling, is essential for maintaining ER homeostasis in pancreatic β-cells. Importantly, IRE1 has a crucial

function in the body's normal production of insulin in response to high glucose. Phosphorylation and subsequent activation of IRE1 by transient exposure to high glucose is coupled to insulin biosynthesis, while inactivation of IRE1 by siRNA or inhibition of IRE1 phosphorylation abolishes insulin biosynthesis. IRE1 signaling under these physiological ER stress conditions utilizes a unique subset of downstream components of IRE1 and has a beneficial effect on pancreatic β-cell homeostasis.

In contrast, we show that chronic exposure of β-cells to high glucose causes pathological levels of ER stress and hyperactivation of IRE1, leading to the degradation of insulin mRNA. The term "glucose toxicity" refers to impaired insulin secretion by β-cells in response to chronic stimulation by glucose and is characterized by a sharp decline in insulin gene expression. However, the molecular mechanisms of glucose toxicity are not well understood. We show that hyperactivation of IRE1 caused by chronic high glucose treatment or IRE1 overexpression leads to insulin mRNA degradation in pancreatic β-cells. Inhibition of IRE1 signaling using a dominant negative form of the protein prevents insulin mRNA degradation in β-cells. Additionally, islets from mice heterozygous for IRE1 retain expression of more insulin mRNA after chronic high glucose treatment than do their wild-type littermates.

This work suggests that the rapid degradation of insulin mRNA could provide immediate relief for the ER and free up the translocation machinery. Thus, this mechanism may represent an essential element in the adaptation of β-cells to chronic hyperglycemia. This adaptation is crucial for the maintenance of β-cell homeostasis and may explain in part why the β-cells of diabetic patients with chronic hyperglycemia stop

producing insulin without simply undergoing apoptosis. This work implies that prolonged activation of IRE1 signaling is involved in the molecular mechanisms underlying glucose toxicity.

This work therefore reveals two distinct activities elicited by IRE1 in pancreatic β-cells. IRE1 signaling activated by transient exposure to high glucose enhances proinsulin biosynthesis, while chronic exposure of β-cells to high glucose causes hyperactivation of IRE1, leading to the degradation of insulin mRNA. Physiological IRE1 activation by transient high glucose levels in pancreatic β cells has a beneficial effect on insulin biosynthesis. However, pathological IRE1 activation by chronic high glucose or experimental drugs negatively affects insulin gene expression. In the future, a system to induce a physiological level of IRE1 activation, and/or reduce the pathological level of IRE1 activation could be used to enhance insulin biosynthesis and secretion in people with diabetes, and may lead to the development of new and more effective clinical approaches to the treatment of this disorder.

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LIST OF ABBREVIATIONS

Abbreviation or symbol	Term
2DG	2-Deoxyglucose
ASK1	apoptosis signaling kinase 1
ATF	activating transcription factor
ATP	adenosine triphosphate
Bcl-2	B-cell leukemia/lymphoma 2
BiP	immunoglobulin heavy chain-binding protein
CHOP	C/EBP-homologous protein
DNA	deoxyribonucleic acid
DTT	dithiothreitol
eIF2α	eukaryotic translation initiation factor 2, α subunit
ELISA	Enzyme-Linked ImmunoSorbent Assay
ER	endoplasmic reticulum
ERO1	ER oxidoreductase 1
ER stress	endoplasmic reticulum stress
FFA	free fatty-acids
GADD	growth arrest and DNA damage inducible gene
Gck	glucokinase
GLP-1	glucagon-like peptide 1
GRP	glucose regulated protein
GSIS	glucose stimulated insulin secretion

IRE1	inositol-requiring enzyme 1
IRS-1	insulin receptor substrate-1
JNK	jun N-terminal kinase
KCl	potassium chloride
KRB	Krebs-ringer bicarbonate buffer
mRNA	messenger ribonucleic acid
PDI	protein disulfide isomerase
PDX-1	pancreatic and duodenal homeobox factor-1
PERK	PKR-like ER kinase
RIA	radioimmunoassay
RNA	ribonucleic acid
RNase	ribonuclease
ROS	reactive oxygen species
RPMI	media from Roswell Park Memorial Institute
SCAEF	stimulus-coupling adaptation to ER folding
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
siRNA	small interfering RNA
T1D	type 1 diabetes mellitus
T2D	type 2 diabetes mellitus
TRAF2	TNF receptor-associated factor 2
TG	thapsigargin

TM	tunicamycin
UPR	unfolded protein response
WFS1	wolfram syndrome 1
WT	wild-type
XBP-1	Xbox-binding protein 1

PREFACE

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CHAPTER I

INTRODUCTION

Diabetes mellitus (DM) is a group of metabolic disorders characterized by abnormally high blood glucose levels. This disease is reaching epidemic proportions worldwide with the predicted number of affected adults increasing from 135 million individuals in 1995 to over 300 million by 2025 [1]. About 90-95% of the diagnosed cases are Type 2 DM, considered a polygenic disease influenced by a number of factors including genetic predisposition, aging, and behavioral issues. Through lifelong vascular complications, diabetes leads to high rates of heart attack, stroke, kidney failure, blindness, and amputations. In the United States, diabetes accounts for over 130 billion dollars of health care costs per year, and is the fifth leading cause of death [2].

The pathogenesis of diabetes involves defective insulin signaling and β-cell dysfunction. Insulin, a hormone produced in the β-cells of the pancreas, is essential for lowering blood glucose levels. Pancreatic β-cells' main function is the production, storage, and regulated secretion of insulin. This process is highly sensitive, so that only small changes in blood glucose concentrations result in altered insulin release. Thus, when blood glucose levels are elevated, such as after a meal, β-cells secrete insulin into the bloodstream where it is transported to the liver, adipose tissue and skeletal muscle. At these target tissues, insulin stimulates glucose uptake into the cells where the glucose is then metabolized to produce energy for cellular functions or converted and stored as glycogen or triglycerides. Under normal conditions, the β-cell always maintains a readily available pool of insulin that can be rapidly secreted in response to changes in blood

glucose level. In patients with diabetes, however, the β-cells produce little or no insulin (Type 1 DM), or have defects in insulin secretion (Type 2 DM). This has two negative implications: 1) glucose builds up to toxic levels in the blood, and 2) the cells of the peripheral tissues are starved of their main source of energy.

Pancreatic β-cell dysfunction is a pathogenic component in the progression of both Type 1 and Type 2 DM [3, 4]. Ever since this important role was recognized, extensive research has been done to try to understand the unique properties of the β-cell and how they contribute to insulin production and secretion in response to glucose. Human pancreatic β-cells are found in the islets of Langerhans, specialized clusters of a few hundred to a few thousand endocrine cells which are anatomically and functionally separate from pancreatic exocrine tissue (which secretes pancreatic enzymes and fluid directly into ducts that drain into the duodenum). Normal individuals have about one million islets, which constitute 1 to 2 percent of the mass of the pancreas. The islets themselves are composed of several types of cells. The majority of the islet (about 80%) is composed of β-cells, which make up the inner core of the islet. These cells are surrounded by α-cells that secrete glucagon, δ-cells that secrete somatostatin, and PP cells that secrete pancreatic polypeptide [5, 6].

In order to compensate for insulin release and maintain sufficient intracellular stores of insulin, β-cells must constantly adjust their rates of insulin biosynthesis. Biologically active insulin is first synthesized in the cytoplasm as a precursor molecule called preproinsulin. Preproinsulin mRNA contains a signal peptide that directs it across the endoplasmic reticulum (ER) membrane through interactions between the signal

peptide and the signal-recognition particle. After its cotranslational translocation into the ER lumen, the signal peptide is cleaved, producing proinsulin. In the lumen of the ER, proinsulin undergoes meticulous processing and folding so that three critical disulfide linkages are correctly aligned. This process is catalyzed by protein disulfide isomerase (PDI) , ER oxidoreductase α (ERO1α) and the molecular chaperone BiP [7, 8]. Once past the ER's internal quality control system, proinsulin is transferred to the Golgi apparatus and packaged into secretory granules. It is inside these granules that the proinsulin is converted into the mature form of insulin. The granules keep insulin in crystalline form in an intracellular storage pool until eventually triggered to transport it to the plasma membrane for exocytosis. The insulin content of the β-cell stays relatively constant under normal conditions, striking an important balance between secretion and biosynthesis and ensuring a readily available, rapidly releasable pool of insulin [7].

Signals activating glucose-stimulated proinsulin biosynthesis in response to glucose-stimulated insulin secretion are, unfortunately, unknown. It has been shown that glucose metabolism is required, but secondary signals beyond that remain to be determined. The proinsulin biosynthetic machinery's response to an increase in glucose concentration is rapid [9] and for short term stimulation (less than 2 hours) seems to be regulated entirely at the translational level in that it does not alter the total amount of preproinsulin mRNA [10, 11]. The rapid response to glucose uses translational regulation of pre-existing preproinsulin mRNA. Interestingly, the translational response of the β-cell to increased glucose concentration seems to be specific for proinsulin biosynthesis[12]. General protein synthesis translation is only increased at most, two-fold

by high glucose concentrations, whereas glucose-induced proinsulin biosynthesis can be increased 15-fold above general protein synthesis [13, 14]. Thus, proinsulin translation accounts for the majority of protein synthesis in glucose stimulated cells.

During longer glucose stimulation periods (greater than 4 hours) transcriptional upregulation of insulin has also been observed [15]. Indeed, chronic elevation of extracellular glucose concentrations further stimulates the biosynthesis of insulin by increasing expression of preproinsulin mRNA, increasing proinsulin translation and processing, and upregulating the capacity of the β-cell's secretory pathway [16]. Both acute and chronic hyperglycemia stimulate β-cells to secrete insulin. This release of insulin simultaneously activates insulin biosynthesis in the ER of the β-cells. β-cells must therefore adapt their acute and chronic rates of insulin biosynthesis to compensate for insulin release. Any imbalance between the physiological load of insulin translation into the ER and the actual capacity of the ER to fold and process the insulin can negatively affect the homeostasis of β-cells and lead to ER stress [17].

ER stress is a specific type of intracellular stress caused by misfolded or unfolded proteins accumulating in the ER[18, 19]. ER stress is broadly defined as any perturbation that compromises the protein folding functionality of the ER, including mutation of ER chaperones or foldases, viral infections, exposure to chemical agents, metabolic demands, and importantly, the normal differentiation and function of secretory cells [20, 21]. The ability to adapt to physiological levels of ER stress has been suggested to be especially important for secretory cells, because the ER is subject to greater loads of client proteins which must be properly folded by the protein processing machinery [17]. This holds true

for pancreatic β-cells, which as professional secretory cells, must fold and process large amounts of insulin [22]. The frequent fluctuation of blood glucose levels requires that β-cells control insulin folding with exquisite sensitivity in order to maintain intracellular insulin stores. Diabetes may result when the β-cell fails to adapt to ER stress caused by the increased demand for insulin production imposed by high blood glucose concentrations.

Cells respond to ER stress via an intracellular adaptive response called the unfolded protein response (UPR). In metazoans, three distinct ER-resident transmembrane proteins have been identified as the upstream transducers of the UPR and are constitutively expressed. Inositol-requiring-1 (IRE1) was the first UPR master regulator to be identified initially in yeast [23, 24]. The unique functions of IRE1 have been conserved in all eukaryotic cells, but higher eukaryotes also possess the additional UPR transducers protein kinase RNA (PKR)-like ER kinase (PERK), and activating transcription factor-6 (ATF6). Each of these three upstream transducers sense the protein folding status in the lumen of the ER and transmit this information across the ER membrane into the cytoplasm. The mechanisms of exactly how they sense the accumulation of unfolded proteins is still not well described, however, all three appear to be activated in part by the competitive titration of the ER chaperone BiP away from interactions with themselves in favor of binding to unfolded proteins in the lumen [25-27].

The UPR utilizes three main responses to mitigate ER stress. The first is a transient adaptation designed to immediately reduce the protein workload of the

overwhelmed ER through reduction in protein synthesis and ER translocation, and an increase in protein degradation. The second is a longer term adaptation designed to increase the capacity of the ER through transcriptional upregulation of UPR target genes, including ones encoding chaperones, foldases, and other proteins that function as part of the ER protein folding machinery. The final response, apoptosis, may occur if ER homeostasis cannot be restored via the first two mechanisms and is presumably a protective response [28]. Although there appears to be much overlap in the pathways that contribute to the protein folding and degradation capacity of the ER during stress, each of the three upstream transducers of the UPR may uniquely regulate some genes and control some physiological responses [29, 30].

ATF6, an upstream ER stress transducer, is a transcription factor which is synthesized as an inactive molecule bound to the ER membrane by its transmembrane segment. Upon ER stress, BiP dissociation from ATF6 releases ATF6 from the ER membrane where it is then transported to the Golgi apparatus. There it is cleaved by site 1 and site 2 proteases to release the active DNA-binding portion into the cytosol [31]. The active form of ATF6 then goes to the nucleus where it upregulates genes encoding ER chaperones including BiP, GRP94, PDI, and calreticulin [32, 33]. There are two isoforms, ATF6α and ATF6β. It has been recently shown that the α isoform is solely responsible for transcriptional induction of ER chaperones [34]. In addition to regulating this subset of genes in response to acute ER stress, ATF6 is also thought to augment the protective mechanisms upregulated by the PERK and IRE1 pathways in an effort to suppress apoptotic UPR signaling cascades and allow the cell to withstand chronic ER stress [30].

A second ER stress transducer, PERK, is a type 1 ER resident protein that contains a kinase domain in the cytoplasmic portion of the protein. Under ER stress conditions, PERK senses the accumulation of misfolded proteins in the ER lumen and becomes active via BiP dissociation, which allows dimerization and trans-autophosphorylation of PERK [35]. The activated kinase domain then phosphorylates the α subunit of the cytosolic eukaryotic translation initiation factor eIF2, which inhibits assembly of the *80S* ribosome, thereby inhibiting protein synthesis [36, 37]. The result is a global reduction in the load of proteins entering the already overwhelmed ER. Additionally, PERK induced phosphorylation of eIF2α results in the selective translation of mRNAs responsible for normal UPR function. These mRNAs share the feature that they all require the ribosome to "skip" inhibitory upstream open reading frames in order to have their *AUG* codon recognized properly [38]. The best known example of this is the transcription factor ATF4, which is preferentially translated under ER stress conditions when eIF2α is phosphorylated. ATF4 also activates the transcription of genes important for responding to additional stresses other than ER stress, including synthesis of amino acids and redox balance [39, 40]. Additionally, production of the pro-apoptotic transcription factor CHOP/GADD153 requires PERK, eIF2α phosphorylation, and ATF4 [41, 42]. It has been suggested that long term PERK activation may initiate apoptosis via CHOP induction if ER homeostasis is not restored.

ER stress induced activation of PERK is readily reversible, as unregulated phosphorylation of eIF2α would be disastrous to the cell. The exact regulatory mechanisms and the phosphatases involved remain to be determined. However, it has

been shown that phosphorylated eIF2α is negatively regulated by two genes, GADD34 and CReP, which encode the substrate targeting subunits of two independent phosphatase complexes that can dephosphorylate eIF2α. GADD34 is induced by the gene expression system initiated by eIF2α phosphorylation and so functions in a negative feedback loop [43]. CReP is constitutively expressed and is involved in baseline eIF2α dephosphorylation [44]. These proteins and perhaps others ensure that the general translational attenuation implemented by PERK induced eIF2α phosphorylation is lifted as soon as ER homeostasis is restored.

The third and most highly conserved ER stress transducer, IRE1, is a type 1 ER resident transmembrane protein kinase/endoribonuclease. In the absence of ER stress, IRE1 exists as inactive monomers in the ER membrane. Under ER stress conditions, IRE1 senses the presence of unfolded proteins in the ER lumen, which causes dimerization and *trans*-autophosphorylation of its kinase domains. Dimerization can be directly triggered by the binding of unfolded proteins to the IRE1 lumenal domain, and may also involve the release of the chaperone, BiP, from the lumenal domain[25, 45, 46]. *Trans*-autophosphorylation 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 (XBP-1) [47, 48]. IRE1 RNase activity results in the removal of a 26 nucleotide intron from *XBP-1* mRNA. This unique cytoplasmic splicing reaction initiates a translational frameshift which produces a form of XBP-1 that contains a novel transcriptional activation domain.

Spliced XBP-1 is a transcriptional activator that plays an essential role in the activation of a wide variety of UPR target genes, including many genes responsible for ER associated degradation (ERAD) [49]. Levels of *XBP-1* mRNA increase upon induction of the UPR and continue to go up as ER stress is reduced and IRE1 is inactivated. Thus, the new *XBP-1* mRNA remains in its unspliced form, which is also translated and encodes an inhibitor of XBP-1 signaling [50]. This may serve to terminate signaling, either through inhibitory heterodimerization of spliced and unspliced XBP-1 proteins, or competition for binding sites. The IRE1-XBP-1 pathway has been found to have a critical role in the differentiation of B lymphocytes into plasma cells [51, 52]. These studies suggest that IRE1-XBP-1 signaling may be required for the function and differentiation of cells that secrete high levels of protein.

In metazoans, IRE1 has additional signaling activity independent of XBP-1 splicing. If the accumulation of unfolded proteins in the ER is unable to be resolved via the IRE1-XBP-1 pathway, prolonged activation of IRE1 activates stress-induced Jun N-terminal kinase (JNK), which can lead to apoptosis in some cell types [53, 54]. However, aside from indirect evidence for a contribution to death in severe ER-stressed cells, the physiological relevance of JNK activation by IRE1 remains unclear.

Recent work in *Drosophila* cells has shown evidence for more widespread IRE1-dependent degradation of ER-associated mRNAs[55]. This is an XBP-1-independent pathway involving specific cleavage and subsequent degradation of sets of mRNAs being translated on the ER membrane. This response complements other components of the UPR, selectively halting protein synthesis and clearing the translocation machinery when

translating mRNAs are overloading the ER and causing ER stress. It has been suggested that this specific mRNA degradation may result from IRE1 focusing on messages that present the most immediate challenge to the translocation and folding machinery [55].

Collectively, activation of the three upstream UPR transducers, ATF6, PERK, and IRE1 proceeds independently in ER stressed cells, however, all three arms communicate extensively with one another to prevent the accumulation of unfolded proteins. The UPR broadly functions to increase the capacity of a cell to carry out protein secretion by utilizing signaling pathways that ensure that the rate of protein synthesis, folding, and degradation are tightly controlled. Accumulating evidence suggests that protein misfolding in the ER lumen and defects in UPR signaling play significant roles in the etiology of numerous diseases states, including neurodegenerative disorders and metabolic diseases like diabetes.

Pancreatic β-cell dysfunction is a major component in the progression of both Type 1 and Type 2 diabetes. The relationship between ER stress and pancreatic β-cell dysfunction was first revealed in a rare autosomal recessive form of juvenile diabetes, Wolcott-Rallison syndrome. In this syndrome, mutations occur in the EIF2AK3 gene encoding PERK [56]. These mutations occur within the catalytic domain of PERK, and cause a loss of function of the kinase activity of PERK and lead to decreased phosphorylation of eIF2α. If a high workload is placed on the ER, eIF2α phosphorylation is essential in mitigating the ER stress level, thereby promoting cell survival [36]. Therefore, the loss of function of PERK and decreased eIF2α phosphorylation may directly lead to β-cell dysfunction or death. Indeed, PERK knockout mice develop

diabetes due to β-cell apoptosis caused by a high level of ER stress in the pancreatic islets [57]. In addition, when mice that have a heterozygous mutation in the phosphorylation site of eIF2α are fed a high-fat diet, they become obese and diabetic due to β-cell dysfunction [58]. Collectively, these observations strongly suggest that regulation of translation initiation through PERK/eIF2α is necessary for ER stress signaling to prevent β-cell dysfunction when the demand for insulin is increased due to a high fat diet and insulin resistance. These studies also imply that β-cells have a unique requirement for PERK regulated translation, and suggest that β-cell dysfunction and death in patients with Wolcott-Rallison syndrome is caused by a high level of ER stress and a defect in the UPR.

The idea that, as a professional secretory cell, the β-cell is particularly vulnerable to ER stress, was further supported by the characterization of the Akita mouse. The Akita diabetes mouse model is a C57BL/6 mouse that is heterozygous for a mutation in the insulin 2 gene [59]. This mutation results in a tyrosine substitution for a highly conserved cysteine involved in the formation of one of the three critical disulfide bonds found in mature insulin protein. This mutation causes incorrect folding of proinsulin in the ER of pancreatic β-cells, and so is toxic due to the induction of the ER stress response, and thus leads to diabetes [60, 61]. The mutant protein cannot be processed and secreted normally and is retained in the ER. The ER in Akita β-cells has been shown to be distended and the UPR response genes BiP and CHOP are upregulated. Deletion of the CHOP gene in these mice delayed the onset of both hyperglycemia and β-cell destruction. Thus, studies of the

Akita diabetes model support the hypothesis that severe ER stress causes β-cell death [62].

More recently, mutations in the human insulin gene INS have been linked to neonatal diabetes [63, 64]. The mutations are in critical regions of the preproinsulin molecule, and are predicted to prevent normal folding and progression of proinsulin in the insulin secretory pathway. The abnormally folded proinsulin may induce the UPR and undergo degradation in the ER, causing severe ER stress, and potentially, β-cell death by apoptosis. One of the human mutations reported in this study is identical to that in the Akita mouse. The identification of insulin mutations as a cause of neonatal diabetes further supports the idea that insulin misfolding in the ER can cause ER stress and lead to β-cell death.

ER stress is also involved in β-cell death in patients with Wolfram syndrome. In 1938, Wolfram and Wagener provided the first report of Wolfram Syndrome when they analyzed four siblings with the combination of juvenile diabetes and optic atrophy [65]. Although patients with Wolfram syndrome are not generally obese, the β-cells in their pancreatic islets are selectively destroyed [66]. Families that exhibit Wolfram syndrome share mutations in a gene encoding WFS1 protein, a transmembrane protein in the ER [67, 68]. However, the precise molecular mechanisms underlying β-cell death caused by the WFS1 mutation remain unknown.

Our lab recently found that WFS1 is a component of the UPR, and that it functions in mitigating ER stress in pancreatic β-cells [69]. We also found that WFS1 mRNA and protein are induced by ER stress and that expression of WFS1 is regulated by

IRE1 and PERK. WFS1 is normally upregulated during insulin secretion, whereas the inactivation of WFS1 in β-cells causes a high level of ER stress and, consequently, β-cell dysfunction. The suppression of WFS1 causes an imbalance in ER homeostasis and leads to an increase in the expression of the ER stress markers BiP, CHOP, Ero1α, spliced XBP-1, and total XBP-1 in INS-1 832/13 cells, a rat pancreatic β-cell line. Our lab has recently found that WFS1 negatively regulates the function of XBP-1 and ATF6 (Fonseca, S. and Urano, F., unpublished data). This finding indicates that WFS1 has an important function in mitigating high levels of ER stress and maintaining ER homeostasis in pancreatic β-cells, suggesting that β-cell death in Wolfram syndrome is caused by chronic and high levels of ER stress. WFS1 knockout mice also develop diabetes due to β-cell apoptosis induced by ER stress [70]. These results indicate that the pathogenesis of Wolfram syndrome involves chronic ER stress in pancreatic β-cells caused by the loss of function of WFS1.

In addition to these rare genetic forms of Type 1 diabetes, emerging evidence suggests that all three upstream signaling components of ER stress, PERK, ATF6, and IRE1, may play important roles in the progression to type 2 diabetes mediated by ER stress. Regulation of translation initiation through PERK/eIF2α is necessary for ER stress signaling to prevent β-cell dysfunction when the demand for insulin is increased due to high fat diet and insulin resistance [58]. These studies also show that β-cells have a unique requirement for PERK regulated translation, and suggest that β-cell dysfunction and death can be caused by a high level of ER stress. More recently, it was shown that common variants in the ATF6 gene are associated with elevated glucose levels in the

general population, and are significantly associated with type 2 diabetes and disturbed glucose homeostasis [71]. However, the physiological functions of ATF6 in pancreatic β-cells are not yet known.

It has been shown that in the presence of chronic ER stress, IRE1 activates JNK through ASK1 and elicits apoptosis in neurons [72] . This pathway may block the functions of the anti-apoptotic BCL2 family members Bcl2 and Bcl-XL by phosphorylating them, thus causing apoptosis in β-cells. It also is possible that this pathway enhances the functions of two proapoptotic factors, Bax and Bak, and causes apoptosis [73]. Additionally, it recently has been suggested that activation of IRE1-JNK signaling is an important contributor to insulin resistance in the liver cells of patients with Type 2 diabetes. Obesity causes ER stress in the liver and leads to hyperactivation of IRE1-JNK signaling [74]. This, in turn, causes serine phosphorylation of insulin receptor substrate-1 (IRS-1) and inhibits insulin action in liver cells. Therefore, a high level of ER stress in liver cells contributes to the development of insulin resistance in Type 2 diabetes. Although it is not clear what causes ER stress in the liver, one possibility is that the baseline level of ER stress is high in hepatocytes, as it is in pancreatic β-cells, and that those cells are susceptible to additional ER stress. Taken together, these studies implicate high levels of ER stress in the progression to diabetes.

The major characteristic of Type 2 diabetes is peripheral resistance to the action of insulin; this resistance leads to a compensatory prolonged increase in insulin biosynthesis and secretion. However, hyperglycemia and Type 2 diabetes only develops in subjects that are unable to sustain the β-cell compensatory response [75]. The failure of

β-cells to compensate for the increased demand for insulin caused by insulin resistance is progressive, particularly occurring after the onset of hyperglycemia. This leads to deterioration in β-cell function and eventual loss of β-cell mass from apoptosis [76]. During the β-cell's compensatory attempt to increase insulin production, the folding capacity of the β-cell ER is overwhelmed. Thus, peripheral resistance to insulin activates ER stress-signaling pathways. Studies have shown that compensation fails in subjects that have "susceptible" as opposed to "robust" β-cells [77]. β-cell susceptibility is not well understood but is determined by a combination of genetic and environmental factors. For this reason, chronic ER stress in β-cells may lead to β-cell death in patients with Type 2 diabetes who are genetically susceptible to ER stress or have a defect in ER stress signaling (i.e. UPR).

When we began this work, some genetic evidence existed that implicated ER stress and defective ER stress signaling in the development of rare forms of experimental and clinical diabetes. This evidence suggested that the insulin-secreting β-cell may be especially sensitive to the adverse effects of perturbed ER function. Compared to other differentiated cells, the β-cell requires periodic increases in its capacity to fold and secrete insulin in response to increases in blood glucose. Mutations or deletions of the ER stress transducer PERK had been implicated in β-cell dysfunction due to the inability to downregulate protein biosynthesis in response to increased insulin production. These studies implied both a physiological and pathological role for ER stress signaling in pancreatic β-cells. Preliminary work had shown that both PERK, and the functionally similar IRE1, were upregulated in pancreatic β-cells under normal conditions, however

the role of IRE1 in these cells had not been studied extensively. Because PERK signaling had been proposed to play an important role in β-cell homeostasis and insulin biosynthesis, we wanted to further investigate the role of IRE1 in these processes.

Additionally, the revelation that the IRE1-XBP-1 signaling pathway was critical for the differentiation of plasma cells lead to the hypothesis that ER stress signaling may play an essential physiological role in the function of many different types of professional secretory cells. Thus we asked the question, what are the physiological and pathological roles of ER stress signaling in pancreatic β-cells? This information could give insight into the normal function of pancreatic β-cells and also shed light onto unknown processes involved in insulin biosynthesis and β-cell homeostasis. Here we show that ER resident protein kinase IRE1 plays an essential role in the positive regulation of insulin biosynthesis and that physiological ER stress has a beneficial effect in pancreatic β-cells. However, we also show that prolonged activation of IRE1 signaling negatively affects β-cells in that it results in a decrease in insulin gene expression. Thus, our work implicates chronic ER stress in the mechanisms of glucose toxicity. Lastly, we provide evidence that the IRE1-mediated decrease in insulin mRNA may be part of a protective mechanism that β-cells have uniquely acquired to cope with chronic high glucose-induced ER stress.

CHAPTER II

PHYSIOLOGICAL ER STRESS HAS A BENEFICIAL EFFECT IN PANCREATIC β-CELLS AND PLAYS A ROLE IN THE REGULATION OF INSULIN BIOSYNTHESIS

Summary

In pancreatic β-cells, the endoplasmic reticulum (ER) is an important site for insulin biosynthesis and the folding of newly synthesized proinsulin. Thus, β-cells have an elaborate ER that functions to maintain ER homeostasis. An imbalance in ER homeostasis caused by high physiological load or pathological conditions elicits ER stress. The unfolded protein response (UPR) is an intracellular adaptive response to ER stress. Inositol Requiring 1α (IRE1α), an ER-resident transmembrane protein kinase, is an upstream component of the UPR and a central regulator of ER stress signaling. Here we show that IRE1α has a crucial function in insulin biosynthesis. IRE1α phosphorylation is coupled to insulin biosynthesis in response to transient exposure to high glucose; inactivation of IRE1α signaling by siRNA or inhibition of IRE1α phosphorylation using a dominant negative kinase inactive mutant hinders insulin biosynthesis. IRE1α activation by acute exposure to high glucose does not accompany XBP-1 splicing or BiP dissociation but does upregulate a subset of UPR target genes such as ERO1α and WFS1. Thus, IRE1 signaling activated by transient exposure to high glucose utilizes a unique subset of downstream components and has a beneficial effect in pancreatic β-cells. In contrast, chronic exposure of β-cells to high glucose causes ER stress and hyperactivation of IRE1, leading to the suppression of insulin gene expression.

Introduction

Pancreatic β-cells are specialized for the production and regulated secretion of insulin to control blood glucose levels. In the presence of hyperglycemia, pancreatic β-cells secrete insulin from a readily available pool. At the same time, an increase in insulin release activates insulin biosynthesis. The endoplasmic reticulum (ER) plays an important role in the biosynthesis of insulin. The frequent fluctuation of blood glucose levels in humans requires that β-cells control proinsulin folding in the ER with exquisite sensitivity. Any imbalance between the physiological load of insulin translation into the ER and the capacity of the ER to properly fold and process the insulin negatively affects the homeostasis of β-cells and leads to ER stress [17, 78].

The unfolded protein response (UPR) is an intracellular system that mitigates ER stress. It has been demonstrated that Inositol Requiring 1 (IRE1), an ER-resident transmembrane protein kinase, is an upstream component of the UPR and a central regulator of UPR-specific downstream gene expression. IRE1 senses the presence of unfolded and misfolded proteins in the ER, which causes dimerization, trans-autophosphorylation and subsequent activation of IRE1. Activated IRE1 then splices X-box binding protein-1 (XBP-1) mRNA, which leads to synthesis of the active transcription factor XBP-1 and upregulation of UPR genes. These include ER-associated protein degradation genes and genes important for protein folding [47, 79, 80] IRE1-XBP-1 signaling is also important for expansion of the ER, which in turn, is required for the differentiation of secretory cells [51, 52, 81]. If the overload of unfolded proteins in

the ER is not resolved via the UPR, prolonged activation of IRE1 causes activation of JNK kinases, which can lead to apoptosis[53, 72].

Mammalian cells have two isoforms of IRE1, α and β. IRE1α is expressed ubiquitously, with high levels of expression in the pancreas and placenta [82] while IRE1β is expressed only in epithelial cells of the gastrointestinal tract [83, 84]. It has been shown that IRE1-XBP-1 signaling is particularly important in cells that are active in protein secretion, such as antibody-secreting plasma cells, hepatocytes, and exocrine pancreatic cells [47, 51, 52, 81, 85]. Studies in these cells suggested that physiological ER stress may be necessary for proper differentiation and function in secretory cells. However, the IRE1-XBP-1 signaling pathway in endocrine cells, (i.e., pancreatic β-cells) had not been studied extensively, and few physiological inducers of ER stress had been identified. Also, no previous studies had reported that activation of IRE1 signaling pathways had a function in regulated synthesis of secretory proteins.

Most previous studies of ER stress signaling were done with the use of severe ER stress inducing drugs which simultaneously and strongly activate all subpathways of the UPR. While these approaches were critical to the overall understanding of UPR signaling function, we now wanted to further investigate the role of ER stress signaling under more physiologically relevant conditions. What were the roles of ER stress signaling in normal cellular function? Based on the studies showing a unique requirement of plasma cells for IRE1 signaling, we hypothesized that under various physiologically relevant stress conditions, each individual UPR subpathway might provide a unique and specialized role. The β-cell requires periodic increases in its capacity to fold and secrete insulin in

response to acute increases in blood glucose. Thus, we undertook this study to test the idea that acute exposure of pancreatic β-cells to high glucose levels causes physiological amounts of ER stress and activates IRE1α signaling. What follows is our characterization of the IRE1α signaling subpathway and its effects on insulin biosynthesis in pancreatic β-cells. Here we show that acute exposure to high glucose activates IRE1α and a unique subset of downstream components of IRE1α. Under these conditions, IRE1α activation has a beneficial effect, aiding in the enhancement of postprandial proinsulin biosynthesis in pancreatic β-cells. However, we also show that chronic exposure of β-cells to high glucose conditions hyperactivates IRE1α, and correlates with a suppression of insulin gene expression. Our results imply both a physiological and pathological role for ER stress signaling in pancreatic β-cells.

Materials and Methods

Cell Culture and Transfection of Small Interfering RNA

Rat insulinoma cells, INS-1 and INS-1 832/13, and mouse islets were cultured in RPMI 1640 supplemented with 10% FBS. The Cell Line Nucleofector Kit T with the Nucleofector Device (Amaxa Biosystems, Gaithersburg, MD) was used to transfect small interfering RNA (siRNA) for IRE1α into INS1 cells. At QIAGEN (Valencia, CA), siRNAs for rat IRE1α were designed and synthesized: for rat IRE1α, AAGGCGATGATCTCAGACTTT.

Glucose-Stimulated Insulin Secretion

INS-1 832/13 cells were transfected with siRNA for IRE1α (final concentration: 20 nM) and preincubated with 5 mM glucose overnight, then stimulated with 5 mM or 10 mM glucose for 1 hour. We measured the insulin secretion level and cellular insulin content by radioimmunoassay (RIA) (Linco Diagnostics, St. Charles, MO) and calculated the ratio between secreted insulin and cellular insulin content.

ELISA

The ELISA assays were performed for mouse and rat insulin using the 1-2-3 UltraSensitive Mouse Insulin ELISA kit (Alpco Diagnostics, Windham, NH).

Immunoblotting and Immunoprecipitation

Cells were lysed in ice-cold M-PER buffer (PIERCE, Rockford, IL) containing protease inhibitors for 15 minutes on ice then the lysates were cleared by centrifuging the cells at 13,000 x g for 15 min at 4°C. Lysates were normalized for total protein (10 mg per lane), separated using 4%–20% linear gradient SDS-PAGE (Bio Rad, Hercules, CA) and electroblotted. Anti-phospho IRE1α antibody was generated from bulk antiserum by affinity purification followed by adsorption against the nonphospho analog column peptide (Open Biosystems, Huntsville, AL). The peptide sequence for generating the antibody was CVGRH (pS) FSRRSG. This phosphopeptide was synthesized, multi-link-conjugated to KLH, and used to immunize 2SPF rabbits. Rabbit anti-total-IRE1α antibody (B9134) was generated using a peptide, EGWIAPEMLSEDCK. Anti-actin antibody was purchased from Sigma (St. Louis, MO), anti-BiP (GRP78) and anti-PDI antibodies were purchased from Stressgen (Ann Arbor, MI), anti-eIF2α antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), anti-phospho-eIF2α and anti-phospho-JNK antibodies were purchased from Cell Signaling (Danvers, MA), and anti-insulin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and Sigma (St. Louis, MO). For the immunoprecipitation, cells were lysed in ice-cold buffer (20 mM Hepes [pH 7.5], 1% Triton X-100, 150 mM NaCl, 10% glycerol, 1 mM EDTA) containing protease inhibitors for 15 minutes on ice. IRE1α was immunoprecipitated from the lysates with anti-total-IRE1α antibody NY200 [25].

Non-reducing Gel

IRE1α wild-type and knockout fibroblasts were treated with 10 mM dithiothreitol (DTT) for 30 minutes. DTT was then washed out and chased with DMEM media for 0, 5, or 15 minutes. Cells were lysed in ice-cold M-PER buffer (PIERCE, Rockford, IL) containing protease inhibitors for 15 minutes on ice then the lysates were cleared by centrifuging the cells at 13,000 x g for 15 min at 4°C. Lysates were normalized for total protein (10 mg per lane), treated with 100 mM iodoacetamide, and separated using 4%–20% linear gradient SDS-PAGE (Bio Rad, Hercules, CA) and electroblotted. PDI and actin were measured by immunoblot.

Insulin Biosynthesis

INS-1 cells were pretreated with 5 mM glucose in RPMI for 18 hr, then with 2.5 mM glucose in KRB buffer (135 mM NaCl, 3.6 mM KCl, 10 mM Hepes [pH 7.4], 5 mM $NaHCO_3$, 0.5 mM NaH_3PO_4, 0.5 mM $MgCl_2$, 1.5 mM $CaCl_2$) for 1 hour. The cells were stimulated with KRB buffer containing 16.7 mM of glucose for 1 hour. Lysates from those cells were subjected to SDS-PAGE. The active form of IRE1α (i.e., P-Ire1α) was detected by immunoblot analysis with anti-phospho specific IRE1α antibody and cellular expression levels of total IRE1α, insulin, and actin were measured by immunoblot analysis using the same lysates. We also measured the insulin secretion level and cellular insulin content by ELISA or RIA. Proinsulin biosynthesis was analyzed by proinsulin immunoprecipitation of [^{35}S] methionine-radiolabeled INS-1 lysates as described [86-88].

Immunoprecipitated ^{35}S-labeled proinsulin was then subjected to SDS-PAGE and visualized by phosphorimaging.

Islet Isolation from Mouse Pancreata

Mice were anesthetized by intraperitoneal injection of sodium pentobarbital. Pancreatic islets were then isolated by pancreatic duct injection of 500 U/ml of collagenase solution followed by digestion at 37°C for 40 minutes with mild shaking. Islets were washed several times with HBSS, separated from acinar cells on a discontinuous Ficoll 400 gradient, viewed under a dissecting microscope, and hand-selected.

Real-Time Polymerase Chain Reaction

Total RNA was isolated from the cells by the guanidine-thiocyanate-acid phenol extraction method and reverse transcribed using 1 mg of total RNA from cells with Oligo-dT primer. For the thermal cycle reaction, the ABI prism 7000 sequencer detection system (Applied Biosystems, Foster City, CA) was used at 50°C for 2 minutes, 95°C for 10 minutes, then 40 cycles at 95°C for 15 seconds, and at 60°C for 1 minute.

The relative amount for each transcript was calculated by a standard curve of cycle thresholds for serial dilutions of cDNA sample and normalized to the amount of rat actin. The polymerase chain reaction (PCR) was performed in triplicate for each sample, then all experiments were repeated twice. The following sets of primers and Cyber Green (Applied Biosystems) were used for real-time PCR: for mouse actin,

GCAAGTGCTTCTAGGCGGAC and AAGAAAGGGTGTAAAACGCAGC; for mouse BiP, TTCAGCCAATTATCAGCAAACTCT and TTTTCTGATGTATCCTCTT CACCAGT; for mouse WFS1, CCATCAACATGCTCCCGTTC and GGGTAGGCCTCGCCATACA; for mouse Ero1α, GCTCTCTCCAAAGTGCTTCCA and TGCATCCTGAACTTTATTCCCA; for mouse Chop, CCACCACACCTGAAAGCAGAA and AGGTGAAAGGCAGGGACTCA; for mouse total XBP-1, TGGCCGGGTCTGCTGAGTCCG and GTCCATGGGAAGATGTTCTGG; for mouse spliced XBP-1, CTGAGTCCGAATCAGGTGCAG (original CAG sequence was mutated to AAT to reduce the background signal from unspliced XBP-1) and GTCCATGGGAAGATGTT CTGG.

Results

Direct assay of IRE1 activation: generation of a phospho specific IRE1α antibody

Previously, studies of IRE1 activation were assayed indirectly by measuring the splicing of one of its downstream targets, XBP-1. However, the degree to which IRE1 is phosphorylated is a direct measure of the IRE1 activation level. Accordingly, we measured the IRE1 activation level in endocrine pancreatic cells by generating an anti-phospho-specific IRE1α antibody. We tested the specificity of that antibody by immunoblot analysis of wild-type and kinase inactive K599A human IRE1α [82] expressed in COS-7 cells. The antibody specifically detected wild-type IRE1α, which is autophosphorylated upon overexpression, but did not recognize the kinase-inactive mutant K599A (Figure 2.1A, upper panel). The expression of wild-type and kinase inactive K599A IRE1α was confirmed using an antibody that can recognize both phosphorylated and unphosphorylated IRE1α (total IRE1α) [89] (Figure 2.1A, lower panel). As previously reported, the expression level of kinase-inactive IRE1α was much higher than that of wild-type IRE1α [82, 89].

IRE1α is activated by high glucose in pancreatic islets

Using anti-phospho-specific IRE1α antibody, we measured IRE1α activation levels in mouse pancreatic islets, whose primary function in the metabolism of glucose is to secrete insulin and glucagon. Therefore, we studied the effect of glucose on IRE1α activation by treating islets with two different concentrations of glucose, 2.5 mM and 16.7 mM. Treating islets with a high concentration of glucose (16.7 mM) for 1 hour increased the phosphorylation level of IRE1α compared to 2.5 mM (Figure 2.1B left

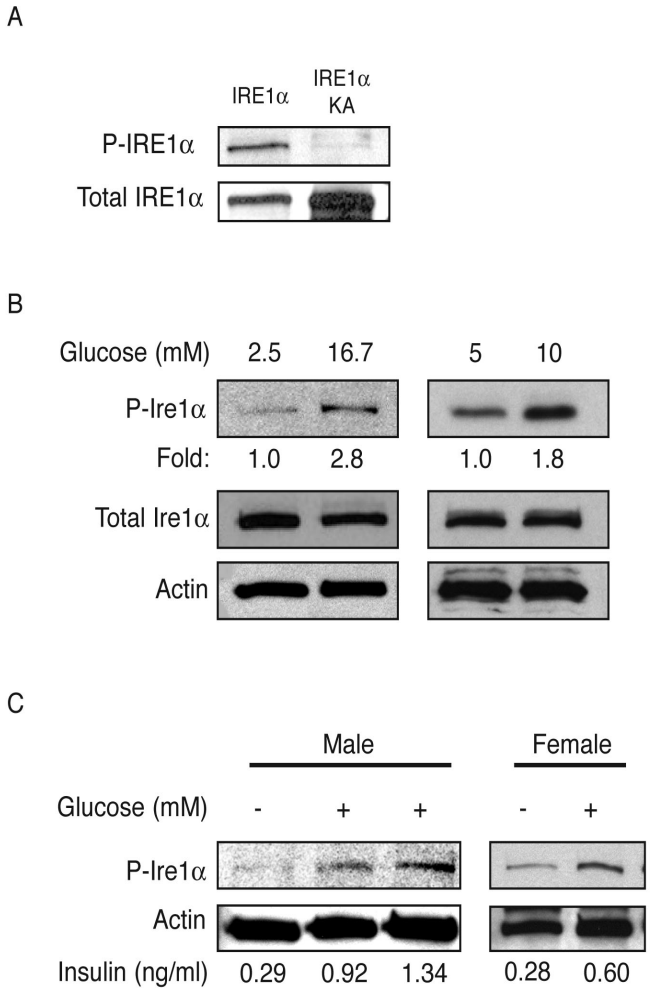

Figure 2.1. IRE1α is activated by high glucose in pancreatic islets
A) PIRE1A1 antibody specifically detects the phosphorylated form of IRE1α protein. Immunoblot analysis of wild-type and kinase-inactive K599A (IRE1αKA) human IRE1α expressed in COS-7 cells using PIRE1A1 antibody (P-IRE1α) or total IRE1α antibody. PIRE1A1 antibody specifically detects wild-type IRE1α, which is autophosphorylated by overexpression. The amount of total IRE1α is shown in the lower panel.

B) Immunoblot analysis of Phospho-Ire1α (P-Ire1α) using lysates from mouse islets treated with 2.5 mM and 16.7 mM glucose (left panel), or 5 mM and 10 mM glucose (right panel) for 1 hour.

C) C57BL/6mice fasted for 14 hours and were then injected intraperitoneally with glucose (2 mg/g) or water. Cellular expression levels of P-Ire1α and actin were determined by immunoblot analysis using the lysates from the islets of these mice. Blood glucose levels, measured by glucometer after 30 min of injection were >350 mg/dL in mice injected with glucose and <150 mg/dL in control mice. Plasma insulin levels were measured by ELISA after 1 hour of injection. Shown is a typical result of an experiment repeated three times.

panel), suggesting that IRE1α is activated in pancreatic islets in the presence of hyperglycemia. Because physiological concentrations of glucose usually fluctuate between 4 mM and 10 mM, we also treated islets with 5 mM and 10 mM glucose for 1 hour and measured IRE1α phosphorylation. Treatment with 10 mM glucose also increased IRE1 phosphorylation (Figure 2.1B, right panel), indicating that IRE1α is activated by high physiological concentrations of glucose. The phosphorylation level of IRE1α by 5 mM glucose was higher than that by 2.5 mM glucose, suggesting that IRE1α activation level is coupled to the glucose concentration. To further study the relationship between glucose concentrations and IRE1α activation, we monitored IRE1α activation levels in vivo. We measured the phosphorylation levels of IRE1α in islets 1 hour after the injection of glucose into C57BL/6 mice. The glucose injection increased plasma insulin levels as expected. In addition, we found that the glucose-stimulated IRE1α phosphorylation and the degree of phosphorylation correlated with the levels of plasma insulin (Figure 2.1C). These results indicate that high glucose treatment activates IRE1α in the pancreatic islets.

To confirm this increase in IRE1α activation by high glucose treatment, we measured mRNA expression levels of ER stress markers in mouse islets treated with 2.5 mM or 16.7 mM glucose for 1 hour using real-time polymerase chain reaction (PCR). Treatment with 16.7 mM glucose caused an increase in the expression of BiP, Wfs1, Ero1α, Chop, and total Xbp-1 (Figure 2A), markers for ER stress. Wfs1 is a target gene of IRE1 signaling in pancreatic β-cells [69]. Therefore, we expected that Xbp-1 splicing would also be activated in islets treated with high glucose. Unexpectedly, Xbp-1 splicing

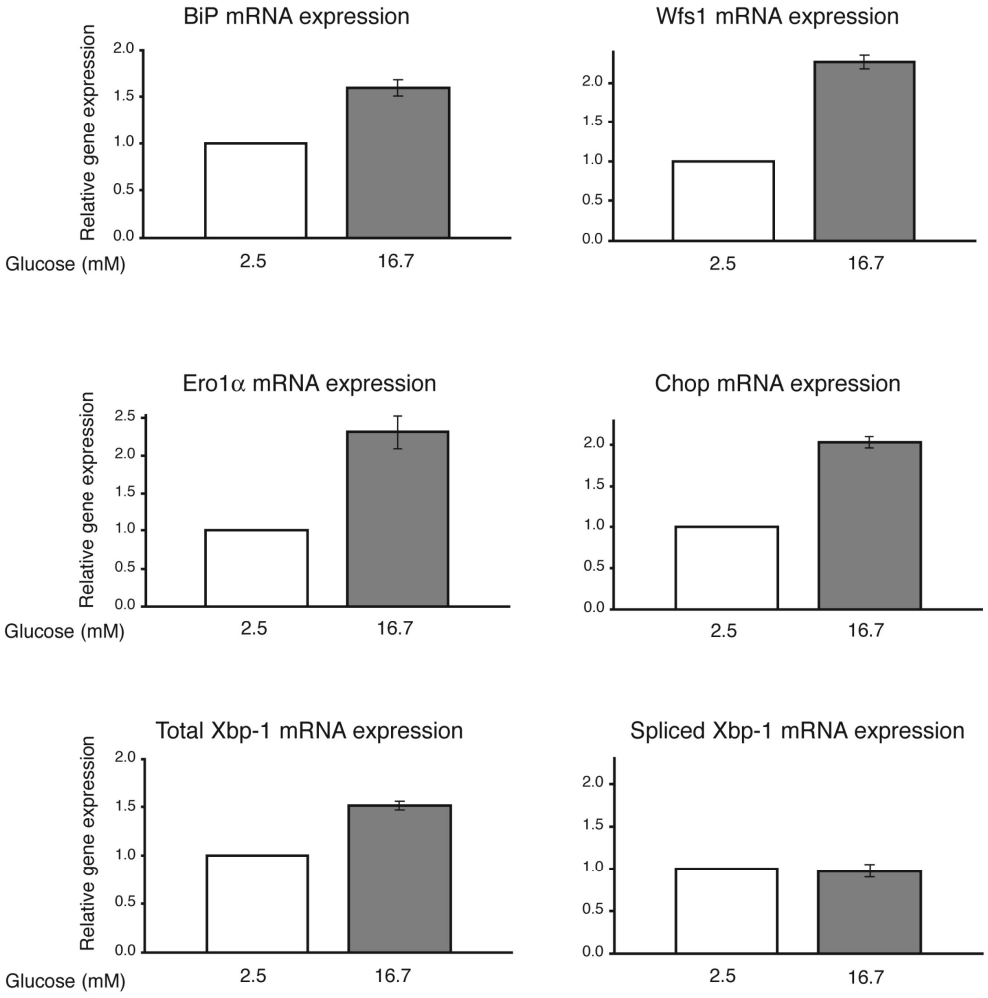

Figure 2.2. IRE1 signaling activated by high-glucose treatment in pancreatic islets upregulates a subset of ER stress response genes

A) Mouse islets were pretreated with 2.5 mM glucose for 1 hour, then treated with 2.5 mM or 16.7 mM glucose for 1 hour. Expression levels of the ER stress markers BiP, Wfs1, Ero1α, Chop, total Xbp-1, and spliced Xbp-1 were measured by real-time PCR (n = 3; values are mean ± SEM).

was not activated by high glucose treatment (Figure 2.2, lowest panel, right side). To confirm this observation, we treated mouse islets with 2.5 mM and 16.7 mM glucose or tunicamycin, a chemical inducer of ER stress for 1 hour, then measured IRE1α phosphorylation by immunoblot. We also measured the expression levels of total Xbp-1 mRNA and spliced Xbp-1 mRNA by real-time PCR. Both high glucose and tunicamycin treatments induced IRE1α phosphorylation and upregulation of total Xbp-1 mRNA (Figure 2.3, top and lower left panels). However, only tunicamycin treatment, and not high glucose treatment, induced Xbp-1 splicing (Figure 2.3 lower right panel). These results suggest that while acute high glucose treatment activates IRE1, this activation may result in signaling independent of the IRE1-XBP-1 signaling pathway.

We hypothesized that the activation of IRE1α caused by transient exposure to high glucose has beneficial effects on the function of the pancreatic islets. The majority of cells in pancreatic islets are insulin producing β-cells. Thus, the activation of IRE1 induced by high glucose in the islets prompted us to study the involvement of IRE1 signaling in insulin biosynthesis in a β-cell line. Through the manipulation of glucose concentration in the insulinoma cell line, INS-1, we found that the IRE1α phosphorylation level increased correspondingly to increases in glucose. Furthermore this increase in IRE1α phosphorylation also correlated with increased insulin secretion by the cells. Treatment of INS-1 cells with high glucose (10, 20, and 25 mM) for 3 hours induced both insulin secretion and IRE1α phosphorylation (Figure 2.4), suggesting that IRE1α may be a positive regulator of insulin secretion and biosynthesis.

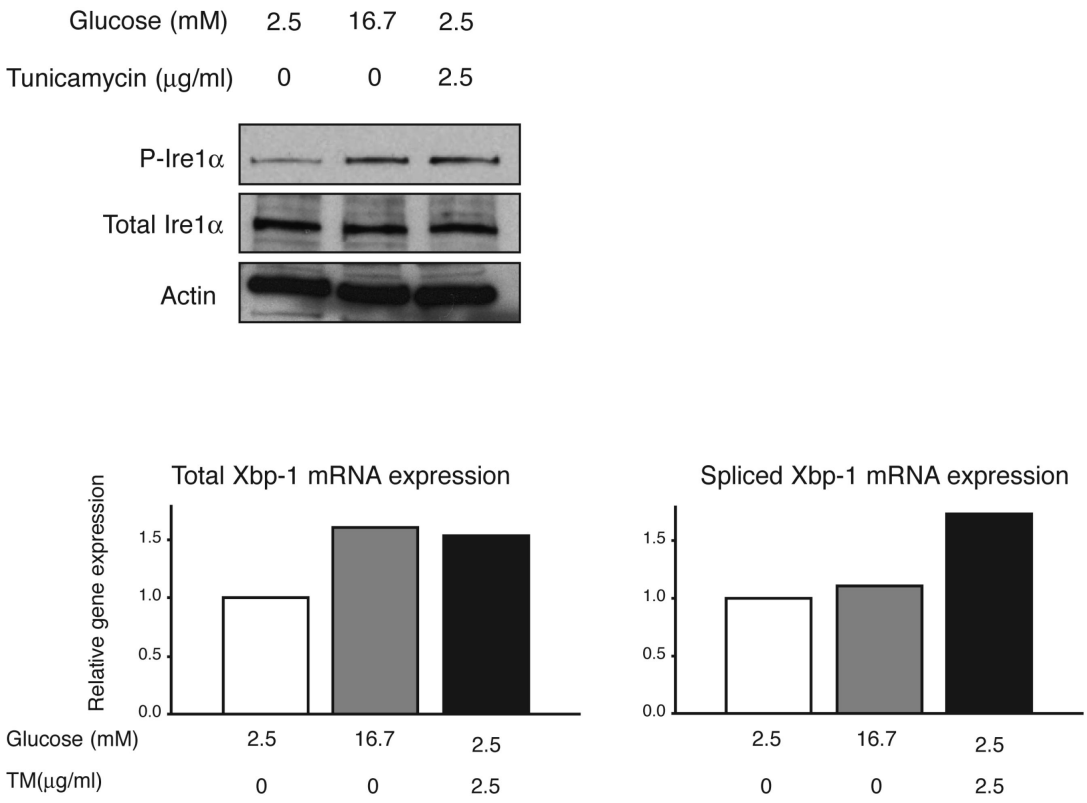

Figure 2.3. IRE1 is activated by both high glucose and ER stress inducing agents but activation by glucose does not accompany XBP-1 splicing

Mouse islets were pretreated with 2.5 mM glucose for 1 hour, then treated with 2.5 mM glucose, 16.7 mM glucose, or tunicamycin (2.5 mg/ml) and 2.5 mM glucose for 1 hour. Expression levels of phospho- Ire1α (P-Ire1α), total Ire1α, and actin were measured by immunoblot. Expression levels of total and spliced Xbp-1 were measured by real-time PCR.

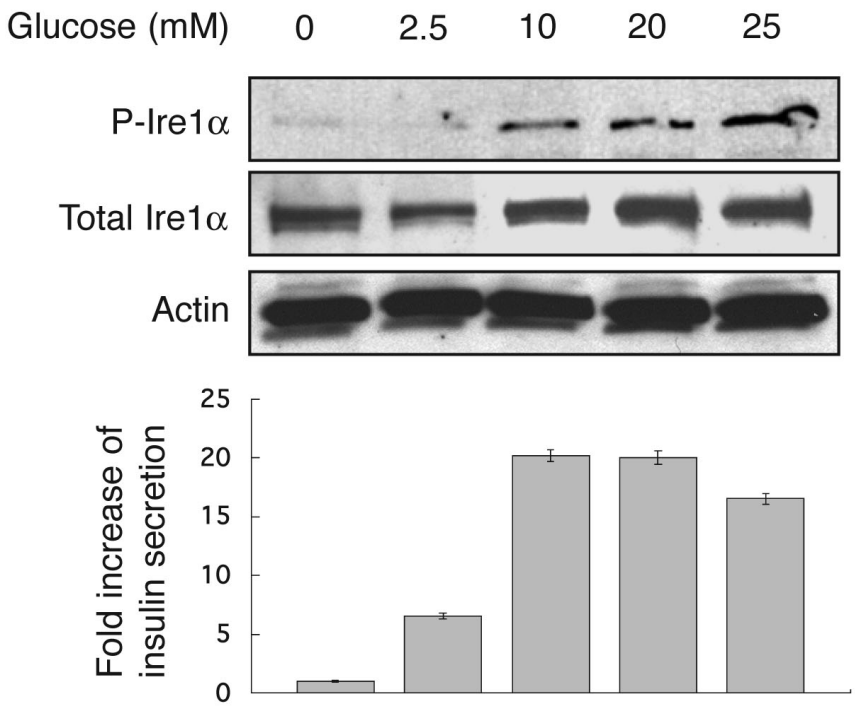

Figure 2.4. IRE1α activation in insulinoma cells corresponds to glucose concentration and insulin secretion

INS-1 cells were pretreated with 5 mM glucose for 18 hour, and then with 2.5 mM glucose for 1 hour. The cells were stimulated with KRB buffer containing 0, 2.5, 10, 20, and 25 mM glucose for 3 hours. Cellular expression levels of phospho-Ire1α (PIre1α), total Ire1α, and actin were measured by immunoblot. The insulin secretion level was measured by ELISA and adjusted to the total amount of protein in cells.

Inhibition of IRE1 signaling decreases insulin biosynthesis

Because IRE1 signaling is important for protein folding in the ER, we hypothesized that IRE1α positively regulates insulin secretion at the biosynthesis level. To study the role of IRE1α in proinsulin biosynthesis directly, we inhibited the expression of IRE1α in INS-1 cells, using siRNA specific for IRE1α. This suppression of IRE1α expression decreased cellular insulin content, but did not affect the expression level of glucokinase (Gck), another important β-cell protein, suggesting that the effects of siRNA are unique to insulin (Figure 2.5A). We also examined the effect of a dominant negative IRE1α mutant, which lacks kinase activity. The expression of mutant IRE1α, in which the ATP binding domain site at lysine residue 599 is replaced with alanine, decreased both the phosphorylation level of endogenous IRE1α and the insulin content in INS-1 cells (Figure 2.5B).

To determine whether IRE1α controls insulin biosynthesis at the translation and protein-folding level, we measured proinsulin biosynthesis in INS-1 cells transfected with siRNA for IRE1α by metabolic pulse-labeling. IRE1α suppression was confirmed by immunoblot with anti-IRE1α antibody (Figure 2.5C, second panel). This suppression of IRE1α dramatically decreased proinsulin biosynthesis, indicating that IRE1α functions in insulin biosynthesis at the translation and protein folding level (Figure 2.5C, top panel). However, IRE1α suppression did not affect the expression levels of the ER proteins, PERK and PDI, or expression of cytoplasmic protein Gck.

To confirm that the suppression of IRE1α by siRNA does not suppress general protein translation, we measured total protein biosynthesis by pulse-labeling, and found

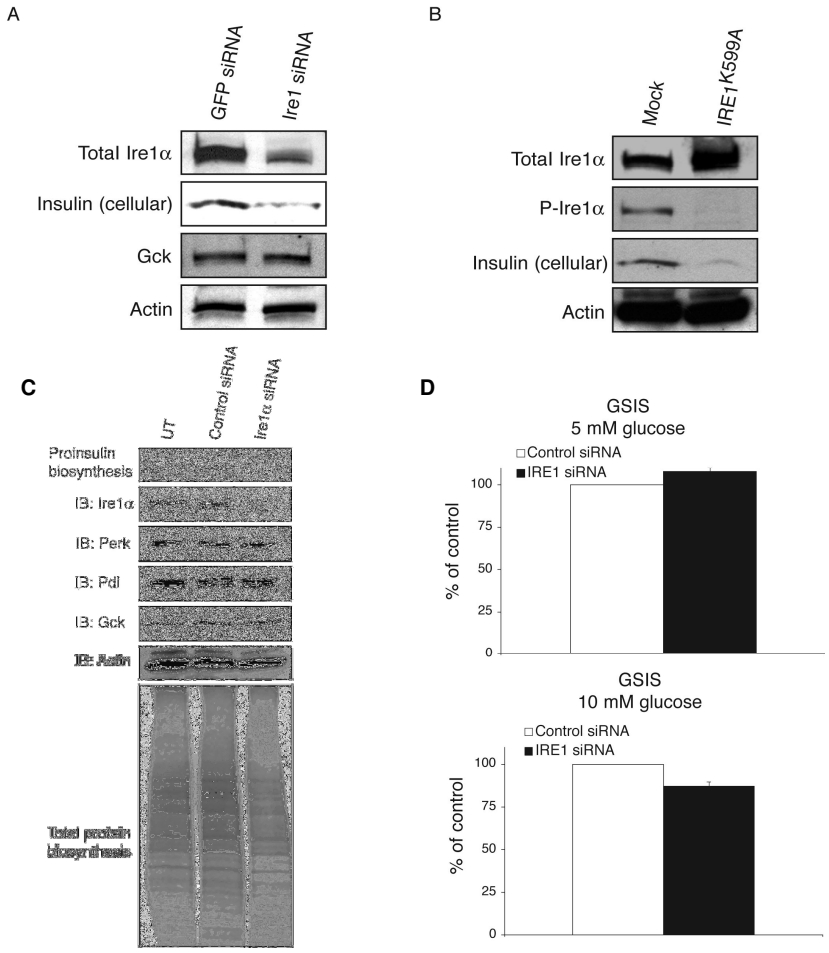

Figure 2.5. Inhibition of IRE1 signaling by siRNA decreases insulin biosynthesis in β-cells
A) INS-1 cells were transfected with siRNA specific for rat Ire1α (final concentration, 20 nM) or siRNA for GFP as a control. Insulin, total Ire1α, glucokinase (Gck), and actin expression levels were analyzed by immunoblot.

B) INS-1 cells were transfected with the kinase-inactive form of IRE1α (IRE1αK599A) or pcDNA3-EGFP. After transfection, the cells were pretreated with 2.5 mM glucose for 18 hours, then with 0 mM glucose for 1 hour. Cells were stimulated with KRB buffer containing 25 mM glucose for 3 hours. Expression levels of total Ire1α, phosphorylated Ire1α (P-Ire1α), insulin, and actin expression were measured by immunoblot.

C) INS-1 cells were transfected with siRNA specific for rat Ire1α (final concentration, 20 nM) or siRNA scramble as a control. After transfection, the cells were pretreated with 11 mM glucose for 18 hours, then stimulated with 16.7 mM glucose and incubated with [^{35}S] methionine for 1 hour. ^{35}S-labeled proinsulin was immunoprecipitated and subjected to SDS-PAGE analysis and visualized with a phosphoimager. ^{35}S-labeled total protein was also analyzed by SDS-PAGE followed by visualization with a phosphoimager. Expression levels of total Ire1α, PERK, PDI, Gck, and actin were measured by immunoblot.

D) INS-1 832/13 cells were transfected with siRNA specific for rat Ire1α or scramble siRNA (final concentration, 20 nM). After transfection, the cells were pretreated with 5 mM glucose for 18 hour, then stimulated with 5 mM glucose or 10 mM glucose for 1 hour. Secreted insulin and cellular insulin content were measured by RIA: the ratio between secreted and cellular insulin (glucose-stimulated insulin secretion, GSIS) was calculated for each sample.

that total protein biosynthesis was not significantly affected by IRE1α siRNA (Figure 2.5C, bottom panel). These results indicate that IRE1α signaling has a beneficial effect on pancreatic β-cells, enhancing insulin biosynthesis. To determine the effect of IRE1α on glucose-stimulated insulin secretion (GSIS: the ratio between secreted insulin and cellular insulin content), we measured GSIS in IRE1α-knockdown β-cells. We transfected INS-1 832/13 cells with siRNA for IRE1α and preincubated the cells with 5 mM glucose overnight, then, stimulated the cells with 5 mM or 10 mM glucose for 1 hour and measured GSIS by radioimmunoassay (RIA). GSIS was not suppressed in IRE1α-knockdown β-cells as compared to control cells with 5 mM glucose (Figure 2.5D, upper panel). GSIS was slightly decreased in IRE1α-knockdown β-cells with 10 mM glucose. These results suggest that IRE1α has an important function in proinsulin biosynthesis, but may not directly affect insulin secretion. Additionally, siRNA knockdown of IRE1α had no effect on insulin gene expression measured by real-time PCR (results not shown), suggesting the beneficial effect of IRE1α activation on insulin production occurs at the translation and protein folding level.

High glucose induced phosphorylation of IRE1α requires glucose metabolism

We next wanted to investigate further the mechanism by which IRE1α is activated in β-cells. To delineate the pathway by which IRE1α is activated by a high level of glucose, we studied the effects of a glucose analog, 2-deoxyglucose (2DG), on IRE1α activation by glucose. By treating INS-1 cells with 5 mM, 10 mM glucose, or 5 mM glucose with 10 mM 2DG, then measuring IRE1α phosphorylation levels, we found that

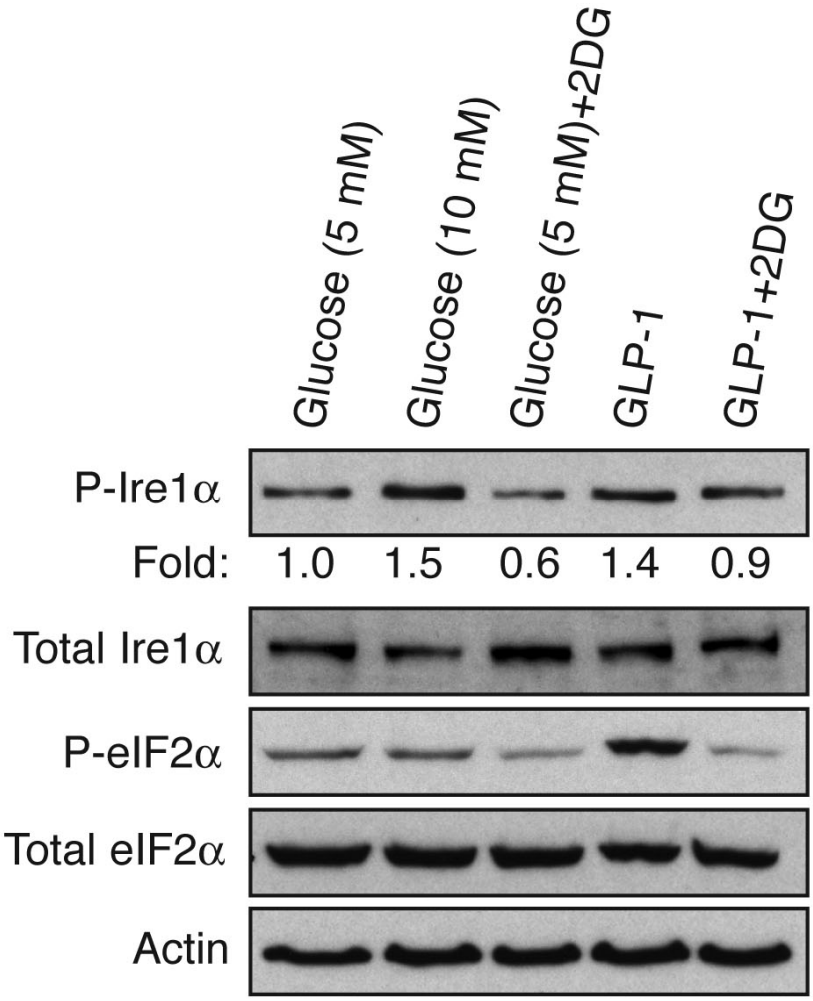

Figure 2.6 IRE1α activation by high glucose requires glucose metabolism
INS-1 cells were pretreated with 5 mM glucose overnight, then stimulated with 5 mM glucose, 10 mM glucose, 5 mM glucose with 10 mM 2-deoxyglucose (2DG), GLP-1 (20 nM) and 5 mM glucose, or a combination of GLP-1 and 5 mM glucose with 10 mM 2DG for 1 hr. Expression levels of phospho-Ire1α (P-Ire1α), total Ire1α, phospho-eIF2α (P-eIF2α), total eIF2α, and actin were measured by immunoblot.

treatment with 2DG decreased IRE1α phosphorylation (Figure 2.6), indicating that glucose metabolism is, in fact, required for IRE1 activation. We also stimulated INS-1 cells with a strong activator for insulin biosynthesis, using GLP-1 and 5 mM glucose as well as a combination of GLP-1 and 5 mM glucose with 2DG. Treatment with GLP-1 and 5 mM glucose increased IRE1α phosphorylation, whereas treatment with a combination of GLP-1 and 5 mM glucose with 2DG prevented the GLP-1 induced increase in IRE1α phosphorylation (Figure 2.6). Because the activation of insulin biosynthesis by GLP-1 requires glucose metabolism, this result supports our finding that glucose metabolism has an important function in IRE1α phosphorylation.

IRE1α activation by insulin secretagogues differs from activation by chemical ER stress inducers

The results from the 2DG treatment studies indicated that IRE1α activation by high glucose required the cells to metabolize the glucose. To test the extent to which IRE1α is activated by other stimuli, we treated INS-1 cells with a variety of known insulin secretagogues, including high glucose, arginine, tolbutamide, KCl, and GLP-1. We saw that all of these agents activated IRE1α phosphorylation (Figure 2.7A). Next, we wanted to compare the difference between IRE1 signaling activated by insulin secretagogues and to that activated by typical ER stress inducers. We treated INS-1 cells with two experimental ER stress inducers, tunicamycin and thapsigargin, and two insulin secretagogues, high glucose and GLP-1. Then, by immunoblot, we measured IRE1α phosphorylation, as well as IRE1's two known downstream effector functions; XBP-1

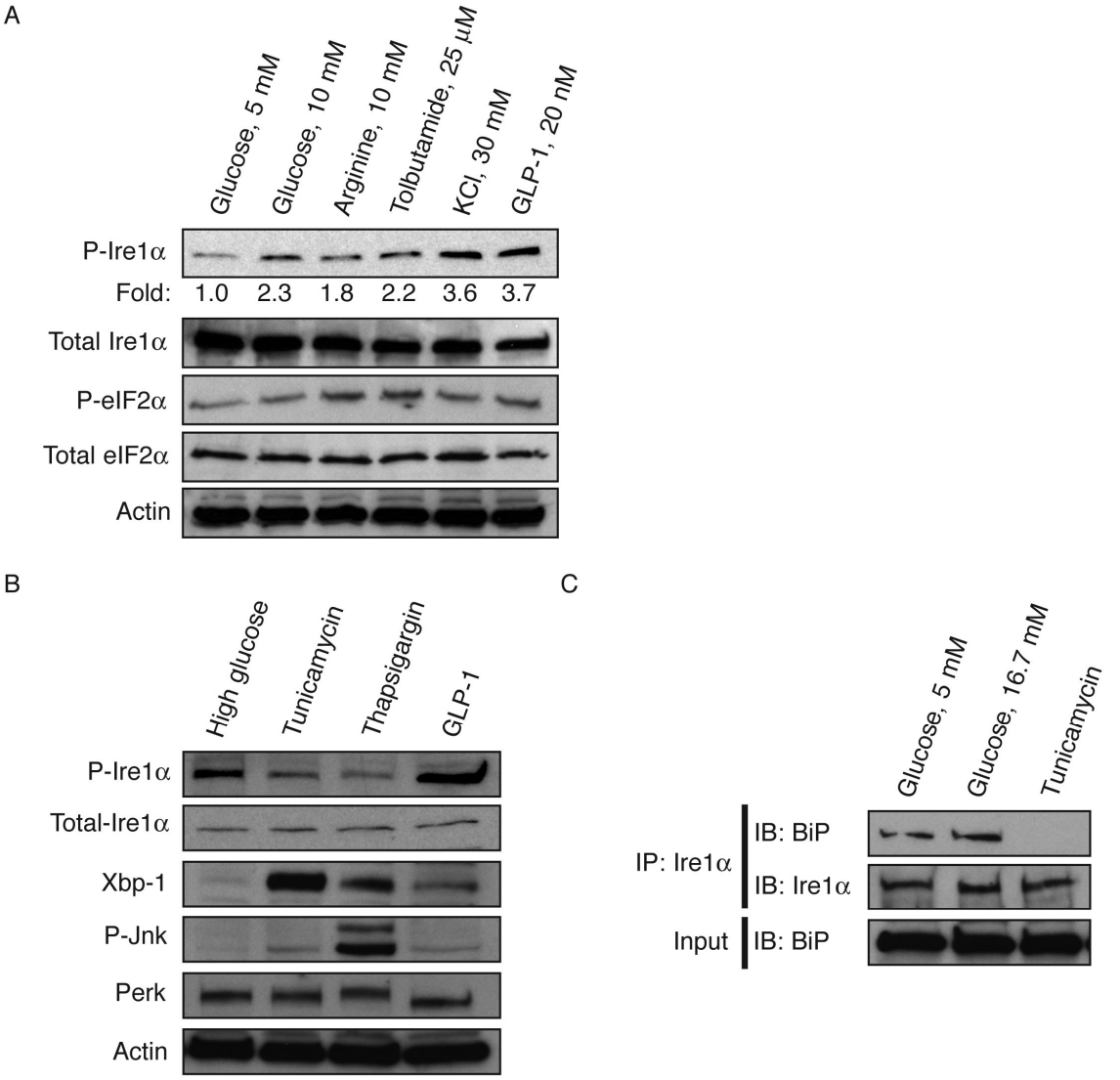

Figure 2.7. The mechanism of IRE1α activation by insulin secretagogues is different from that of activation by chemical ER stress inducers

A) INS-1 cells were pretreated with 5 mM glucose for 18 hours, then stimulated with glucose (5 mM and 10 mM), arginine (10 mM), tolbutamide (25 mM), KCl (30 mM), or GLP-1 (20 nM) for 1 hour. Expression levels of phospho-Ire1α (P-Ire1α), total Ire1α, phospho-eIF2α (P-eIF2α), total eIF2α, and actin were measured by immunoblot.

B) INS-1 cells were pretreated with 5 mM glucose for 18 hours, then stimulated with high glucose (16.7 mM), tunicamycin (2.5 mg/ml), thapsigargin (1 mM), or GLP-1 (20 mM) for 3 hours. Expression levels of phospho-Ire1α (P-Ire1α), total Ire1α, Xbp-1, phospho-Jnk (P-Jnk), Perk, and actin were measured by immunoblot.

C) INS-1 cells were pretreated with 5 mM glucose for 18 hours, then stimulated with 5 mM glucose, 16.7 mM glucose, or tunicamycin (2.5 mg/ml) and 5 mM glucose for 3 hours. Ire1α was immunoprecipitated (IP) using lysates from the cells. IP products were immunoblotted (IB) with anti-BiP antibody.

splicing, and JNK phosphorylation. We also measured PERK expression levels. We found that GLP-1 and high glucose were the strongest activators of IRE1α (Figure 2.7B). However, we also observed that tunicamycin was the strongest activator of XBP-1 splicing and thapsigargin was the strongest activator of JNK phosphorylation (Figure 2.7B). These results show that insulin secretagogues activate IRE1α, but do not strongly activate known downstream components of IRE1 signaling, XBP-1 and JNK. This finding raised the possibility that IRE1α activation by high glucose (a physiological condition) and by tunicamycin and thapsigargin (experimentally induced, pathological conditions) occur by different mechanisms.

It has been shown previously that IRE1 activation is controlled by an ER-resident molecular chaperone, BiP [25]. Under normal, unstressed conditions, BiP binds to the luminal domain of IRE1 and prevents its dimerization and activation. The accumulation of unfolded proteins in the ER (i.e., ER stress) correlates with the engagement of BiP with unfolded proteins and the subsequent release from IRE1, leading to IRE1 dimerization and activation. To study how IRE1α is activated by different stimuli, we treated INS-1 cells with high glucose or tunicamycin, then examined the interaction between IRE1α and BiP by immunoprecipitation. As was reported, we found that BiP dissociated from IRE1α in response to tunicamycin treatment of INS-1 cells (Figure 2.7C). However, BiP remained associated with IRE1α when INS-1 cells were treated with high glucose (Figure 2.7C), showing that IRE1α activation by a high level of glucose differs from its activation by tunicamycin treatment. Specifically, activation by high glucose does not require BiP dissociation.

Transient high glucose treatment causes IRE1 activation and upregulates a unique subset of downstream components of IRE1.

We had observed that acute treatment of β-cells with high glucose caused ER stress and resulted in IRE1α phosphorylation. However, activation of IRE1 with this stimulus did not result in activation of either known downstream signaling target of IRE1α; XBP-1 splicing or JNK phosphorylation. We also observed that this activation of IRE1α had a beneficial effect on proinsulin biosynthesis. We therefore wanted to further investigate how activation of IRE1α exerts its positive effects on insulin folding in the ER. We had shown that ERO1α, an ER-resident oxidoreductase that plays a key role in proinsulin folding, is upregulated by transient high glucose (Figure 2.2.). To investigate if IRE1α was involved in ERO1α expression, we treated WT and IRE1α -/- fibroblasts with the ER stress inducer tunicamycin for increasing time periods (Figure 2.8A). ERO1α mRNA was upregulated with increasing exposure to tunicamycin in the wild-type cells. However, in the IRE1α -/- cells, ERO1α upregulation was significantly reduced, indicating a role for IRE1α in the upregulation of ERO1α under ER stress conditions.

ERO1α is an activator of protein disulfide isomerase (PDI), which has a critical role in catalyzing the formation of disulfide bonds during protein folding in the ER [90-92]. Thus, we hypothesized that the inability of IRE1α -/- cells to upregulate ERO1α in response to ER stress may also impact PDI. We challenged WT and IRE1α -/- cells with the reducing agent DTT, which is a powerful inducer of the ER stress response, for 30

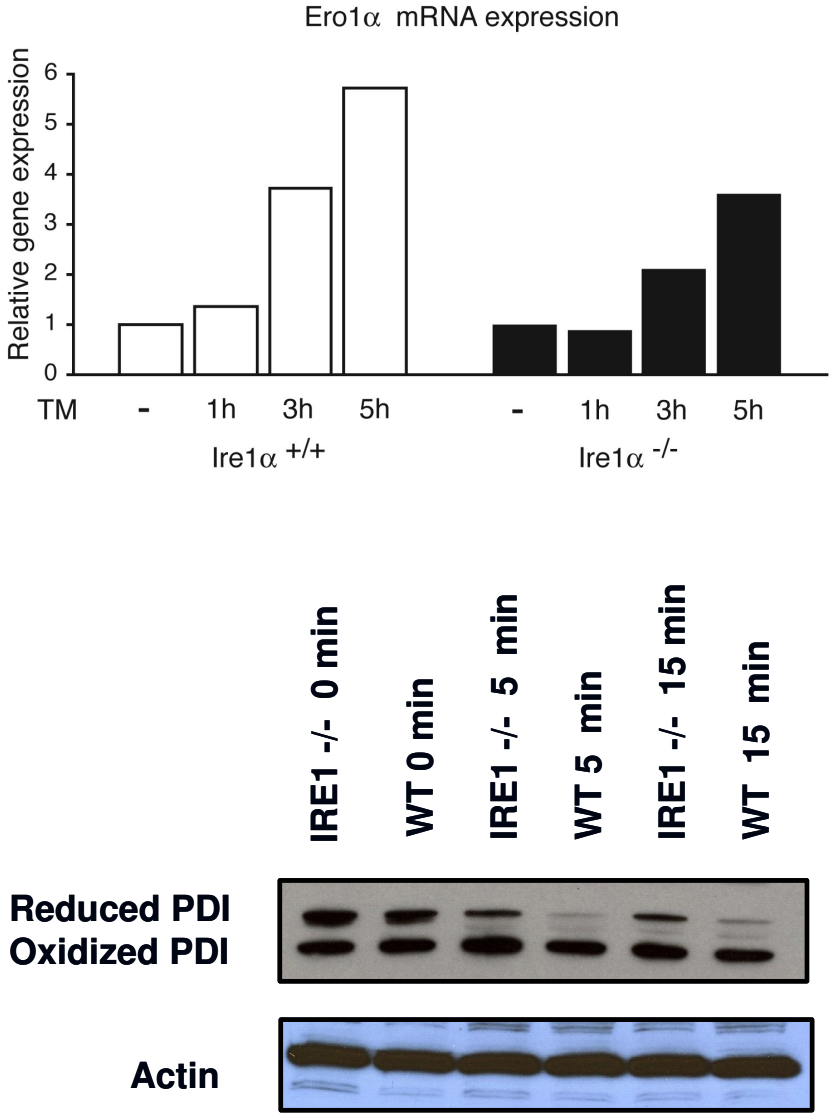

Figure 2.8. IRE1α -/- cells are defective in ERO1α upregulation and PDI re-oxidation when challenged with ER stress

A) IRE1α wild-type and knockout fibroblasts were treated with 2.5ug/ml tunicamycin for 0,1,3,or 5 hours. Expression levels of ERO1α were measured by real-time PCR.

B) IRE1α wild-type and knockout fibroblasts were treated with 10 mM DTT for 30 minutes. DTT was washed out and chased with regular media for 0, 5, or 15 minutes. Lysates were treated with 100 mM iodoacetamide, run on a non-reducing gel, and PDI and actin were measured by immunoblot.

minutes, and then removed the DTT and let the cells recover (Figure 2.8B). We found that the IRE1α -/- cells were defective in the re-oxidation of PDI after DTT removal. Thus, IRE1α might beneficially impact cells though upregulation of ERO1α, which may activate proinsulin biosynthesis by enhancing the formation of the three critical disulfide bonds found in biologically active mature insulin. Because we did not observe XBP-1 splicing or JNK phosphorylation under conditions that activate IRE1α and upregulate ERO1α, we suggest that IRE1α activation by high glucose controls a unique subset of components of IRE1α signaling including ERO1α and PDI.

Sustained IRE1 signaling correlates with decreased insulin gene expression

Treatment of β-cells with high physiological concentrations of glucose for 1 hour enhances proinsulin biosynthesis. In contrast it is known that, paradoxically, chronic hyperglycemia is harmful to this adaptive response. To determine whether chronic hyperglycemia causes the typical activation of IRE1α and ER stress we treated mouse islets for 24 hours with 5 mM, 11 mM, and 16.7 mM glucose, then measured XBP-1 splicing. This exposure of islets to high concentrations of glucose (11 mM and 16.7 mM) did cause XBP-1 splicing, indicating that chronic hyperglycemia induces typical IRE1 activation and ER stress (Figure 2.9A).

We next cultured β-cells with low (2.5 mM), intermediate (11 mM), and high (25 mM) concentrations of glucose for 3 and 7 days, then measured IRE1 activation levels and cellular insulin content to test whether a high level of sustained IRE1 activation(i.e.,ER stress) has a harmful effect on insulin biosynthesis.

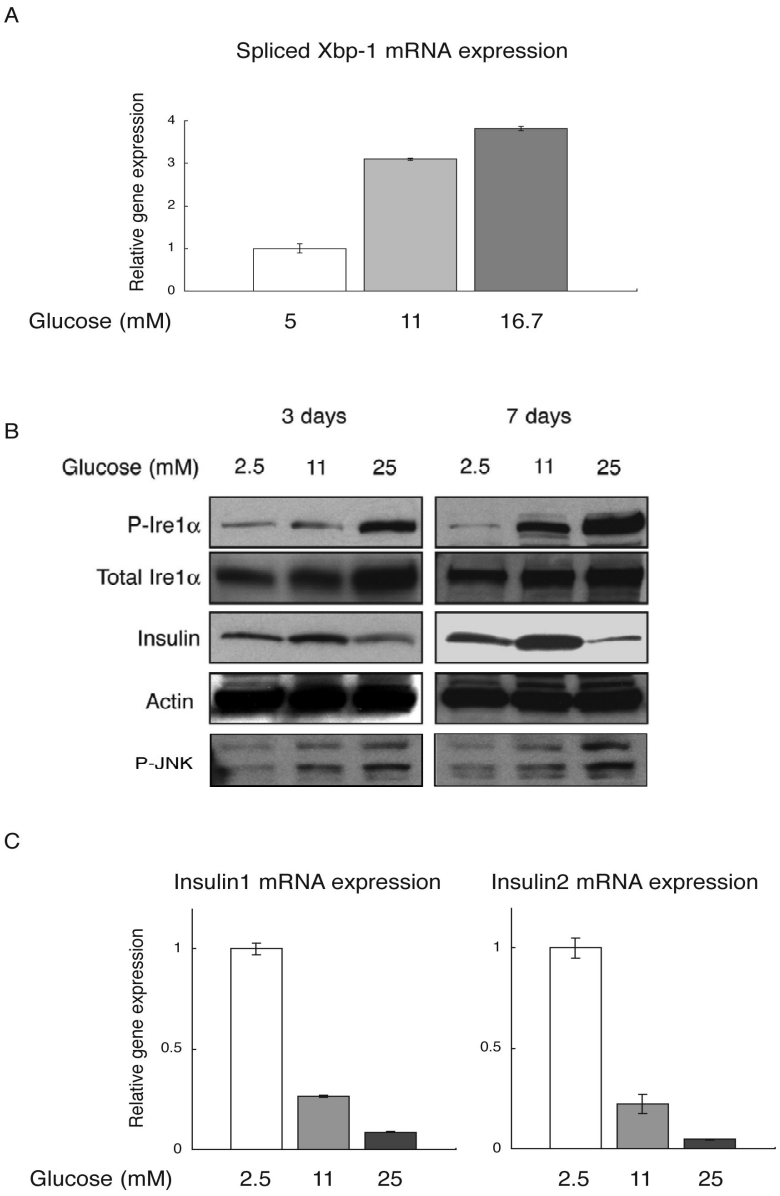

Figure 2.9. Sustained activation of IRE1 signaling decreases insulin gene expression
A) Mouse islets were treated with 5 mM, 11 mM, and 16.7 mM glucose for 24 hours. Spliced XBP-1 mRNA expression levels were measured by quantitative real-time PCR. Error bars represent the SD of duplicate determinations.

B) INS-1 cells were treated with 2.5 mM, 11 mM, and 25 mM glucose for 3 days and 7 days. Expression levels of total Ire1α, phosphorylated Ire1α, insulin, phosphorylated jnk,(both isoforms) and actin were measured by immunoblot.

C) INS-1 cells were treated with 2.5 mM, 11 mM, and 25 mM glucose for 7 days. Insulin 1 (Ins1) and insulin 2 (Ins2) mRNA expression levels were measured by quantitative real-time PCR. Error bars represent the SD of triplicate determinations.

Sustained activation of IRE1α by prolonged high glucose treatment resulted in increased JNK phosphorylation, further suggesting that chronic hyperglycemia may activate typical IRE1α controlled ER stress signaling pathways. Treatment of the cells with 11 mM glucose increased both the phosphorylation level of IRE1α and the cellular insulin content as compared to the levels in cells treated with 2.5 mM glucose (Figure 2.9B). Treatment with 25 mM glucose further increased the phosphorylation level of IRE1α, but decreased the cellular insulin content (Figure 2.9B), suggesting that hyperactivation of IRE1α may decrease insulin biosynthesis. To try to determine why high glucose treatment corresponded to a decrease in cellular insulin content, we also measured the expression levels of rat insulin mRNA in INS-1 cells treated with different concentrations of glucose for 3 days by real-time PCR. The expression levels of insulin mRNA dramatically decreased in the cells treated with 25 mM glucose (Figure 2.9C), suggesting that sustained activation of IRE1α (i.e., chronic ER stress) may decrease insulin biosynthesis at the transcription level. However, these observations may also reflect that the IRE1 activation and the UPR cannot keep up with the damage that chronic hyperglycemia causes.

Discussion

It has been shown that upstream components of ER stress signaling, IRE1 and XBP-1, play important physiological roles in the development of antibody-secreting plasma cells, hepatocytes, and exocrine pancreatic cells [52, 85]. Here, we have shown that regulated activation of IRE1α under physiological settings has an important function in pancreatic β-cells, and add β-cells to the growing list of secretory cells that require components of ER stress signaling for normal day-to-day activity. Postprandial hyperglycemia activates IRE1α, leading to the enhancement of proinsulin biosynthesis. We have therefore determined that glucose is a physiological activator of the UPR in pancreatic β-cells. Since the activation of IRE1 by transient exposure to high glucose does not accompany XBP-1 splicing or JNK activation, we have named this unique biological phenomenon ‘‘Stimulus-Coupling Adaptation to ER Folding (SCAEF)”. The components of SCAEF probably have important functions in proinsulin biosynthesis in pancreatic β-cells. For example, the ER-resident oxidoreductase ERO1α, is upregulated by transient high glucose and functions in proinsulin folding. Since ERO1α is an activator for PDI, which has a crucial role in disulfide bond formation, ERO1α may activate insulin biosynthesis by enhancing disulfide bond formation of proinsulin in the ER. Future studies may benefit greatly from the use of activators and components of SCAEF. These may help reveal different and, perhaps yet unknown, UPR pathways that are not evident through the manipulation of cells with severe ER stress inducing drugs such as tunicamycin and thapsigargin.

The activation of IRE1α by transient exposure to high glucose positively regulates proinsulin biosynthesis. It has been shown that another upstream component of ER stress signaling, PKR-like ER kinase (PERK), is also important in proinsulin biosynthesis [57]. PERK is highly expressed in pancreatic islets [37, 93]. Activated PERK phosphorylates the α subunit of eukaryotic translation initiation factor 2 (eIF2α), leading to the attenuation of general protein translation. This reduces the ER workload and protects cells against apoptosis resulting from ER stress [36]. In islets from PERK knockout mice, insulin biosynthesis stimulated by high glucose is markedly increased as compared to that in control mice, indicating that PERK is needed to suppress insulin biosynthesis by high glucose in β-cells [57]. This observation, combined with our present results, suggests that IRE1α may be a positive regulator and that PERK may be a negative regulator of insulin biosynthesis. Mutations in the EIF2AK3 gene encoding PERK have been reported in Wolcott-Rallison syndrome, a rare form of juvenile diabetes [56]. PERK knockout mice also develop diabetes as a consequence of a high level of ER stress in the pancreas [57]. Thus, the balance between IRE1α and PERK signaling appears to be important in the maintenance of β-cell homeostasis. Indeed, an imbalance between these two pathways may cause β-cell death. PERK and IRE1α may work together to monitor and properly regulate the amount of insulin translation occurring inside the β-cell ER in response to fluctuating blood glucose levels.

Our results also suggest that IRE1 activation by high glucose occurs by a different mechanism than activation by tunicamycin, an extreme ER stress inducer. One current model of IRE1 activation is a ‘‘Competitive Deprivation’’ model of BiP dissociation

[25]. According to this model, BiP binds to the luminal ER domain of IRE1 under normal conditions and prevents dimerization and subsequent activation of IRE1. Under ER stress conditions, BiP engages with unfolded proteins in the lumen and is released from IRE1, leading to IRE1 dimerization and activation. It is known that BiP dissociates from IRE1 under ER stress conditions caused by nonphysiological experimental conditions. Thus, it is possible that under extreme ER stress, BiP needs to be dissociated from IRE1 to fully activate IRE1 signaling, or to signal that conditions in the ER cannot be resolved via the UPR. However, the competitive deprivation model may not account for the adaptation to physiological ER stress caused by frequent fluctuations in protein folding in the ER. Recently, it was shown that the BiP binding domain of IRE1 is not the principal determinant of IRE1 activation [45]. Additionally, another group showed that IRE1 can be directly activated by unfolded proteins in the ER, and that BiP binding and release is not a requirement for control of IRE1 activity [46]. They suggested that BiP binding to IRE1 may serve to dampen activation of downstream IRE1 signaling targets (i.e., XBP-1 or JNK) under conditions of mild ER stress [46]. Our finding of IRE1 activation without BiP dissociation by high glucose supports this possibility.

Physiological activation of IRE1 (i.e., SCAEF) is unlikely to activate apoptosis signaling pathways. This conclusion is supported by our finding that high-glucose activation of IRE1 does not activate JNK signaling pathways, which in certain cell types are proapoptotic components of the UPR. However, we were surprised to find that XBP-1 also is not spliced by transient high-glucose treatment either, indicating that IRE1-XBP-1 signaling is not essential for insulin folding in β-cells under certain physiological

conditions. These observations are supported by work from the Glimcher laboratory showing that XBP-1 knockout mice have a severe exocrine pancreatic phenotype, whereas islets from these same mice are indistinguishable from wild-type islets [85]. It is possible that in endocrine pancreatic tissue, IRE1α has other target mRNAs that are subject to its endonuclease activity. These mRNAs may compete with XBP-1 mRNA so that XBP-1 is not spliced under physiological conditions when glucose is high and the β-cells need to process large quantities of proinsulin. The physiological relevance of this mechanism, however, remains to be determined and requires further investigation.

We have shown that IRE1 has important physiological functions in pancreatic β-cells: it is activated in response to high glucose and positively regulates proinsulin biosynthesis. Nevertheless, IRE1 activation could have a pathological effect under chronic pathological conditions or in other tissues. The major abnormality in patients with Type 2 diabetes is peripheral resistance to the action of insulin, which leads to a prolonged increase in insulin biosynthesis in response to elevated glucose levels. The secretion capacity of the ER is then overwhelmed, causing prolonged activation of the IRE1 signaling pathway. This could lead to glucose toxicity associated with hyperglycemia and insulin resistance. Glucose toxicity is defined as nonphysiological and potentially irreversible β-cell damage caused by chronic exposure to supraphysiological glucose concentrations [94, 95]. Our observation regarding IRE1 hyperactivation and the suppression of insulin biosynthesis suggests that chronic ER stress is a cause for glucose toxicity. However, this observation may also reflect that the stress response cannot keep up with the damage that chronic hyperglycemia causes. More studies are needed to

determine the relationship between IRE1 hyperactivation (i.e., ER stress) and glucose toxicity.

In addition, activation of IRE1-JNK signaling is an important contributor to insulin resistance in the liver cells of patients with Type 2 diabetes. Obesity causes ER stress in the liver and leads to hyperactivation of IRE1-JNK signaling [74] which, in turn, causes serine phosphorylation of insulin receptor substrate-1 (IRS-1) and inhibits insulin action in liver cells. Therefore, a high level of ER stress in liver cells could contribute to the development of insulin resistance in patients with Type 2 diabetes. It has also been shown that a strong IRE1-JNK activation by the accumulation of expanded polyglutamine repeats seen in Huntington disease induces neuronal cell death[96]. These findings indicate that activation of IRE1 by pathological conditions, such as obesity, polyglutamine accumulation, and chronic hyperglycemia, is harmful to cells.

In this study, we observed that chronic exposure to high glucose hyperactivates IRE1 and activates JNK. This activation of IRE1-JNK signaling in β-cells corresponds to reduction in insulin gene expression and cellular insulin content. Our results further support the idea that a high level of ER stress plays a pathological role in β-cell dysfunction. In contrast, we also show that physiological IRE1 activation by transient high glucose levels in pancreatic β-cells has a beneficial effect. Therefore, a drug or system to induce a physiological level of IRE1 activation could be used to enhance insulin biosynthesis and secretion in patients with diabetes, and could lead to the development of new and more effective clinical approaches to the treatment of this disorder.

CHAPTER III

PATHOLOGICAL ER STRESS NEGATIVELY AFFECTS THE HOMEOSTASIS OF PANCREATIC β-CELLS AND RESULTS IN THE REDUCTION OF INSULIN GENE EXPRESSION

Summary

The endoplasmic reticulum (ER) is a cellular compartment that functions in protein folding. Dysregulation of ER homeostasis elicits ER stress and subsequently activates cell signaling pathways, collectively known as the unfolded protein response (UPR). IRE1α is a central component of the UPR. In pancreatic β-cells, IRE1α also functions in the regulation of insulin biosynthesis.

The term "glucose toxicity" refers to the harmful metabolic effects of chronic hyperglycemia. Glucose toxicity is an impairment of insulin production and secretion in response to hyperglycemia and the underlying mechanisms responsible remain to be determined. Accumulating evidence has implicated JNK signaling in oxidative stress mediated suppression of insulin gene expression (i.e., glucose toxicity) in pancreatic β-cells. Here we show that IRE1α signals through the JNK pathway in response to ER stress. In β-cells, ER stress activates IRE1α and correlates with a reduction in nuclear PDX-1. Inhibition of the IRE1α-JNK signaling pathway partially restores PDX-1 protein to the nucleus of pancreatic β-cells. Activation of the IRE1-JNK pathway by chronic high glucose treatment leads to increased serine phosphorylation of PDX-1, suggesting a potential mechanism of PDX-1 cytoplasmic relocalization.

Additionally, we show that hyperactivation of IRE1α caused by chronic high glucose treatment or IRE1α overexpression leads to insulin mRNA degradation in pancreatic β-cells. Inhibition of IRE1α signaling using its dominant negative form prevents insulin mRNA degradation in β-cells. Islets from mice heterozygous for IRE1α retain expression of more insulin mRNA after chronic high glucose treatment than do their wild-type littermates.

These results reveal a role of IRE1α in insulin mRNA expression under ER stress conditions caused by chronic high glucose. The rapid degradation of insulin mRNA could provide immediate relief for the ER and free up the translocation machinery. The slower transcriptional downregulation through PDX-1 inactivation may further ease the stress of translocating insulin. Thus, this mechanism would preserve ER homeostasis and help ensure that the insulin already inside the ER can be properly folded and secreted. This adaptation may be crucial for the maintenance of β-cell homeostasis and may explain why the β-cells of type 2 diabetic patients with chronic hyperglycemia stop producing insulin without simply undergoing apoptosis. This mechanism may also be involved in suppression of the autoimmune diabetes (Type 1 diabetes) by reducing the amounts of misfolded insulin, which could be a source of "neo-autoantigens."

Introduction

The endoplasmic reticulum (ER) is a cellular compartment for the biosynthesis and folding of newly synthesized secretory proteins such as insulin. This environment in the ER is sensitive and can be perturbed by both pathological processes (viral infections) and physiological processes (large biosynthetic load placed on the ER). This perturbation in ER function causes disregulation of ER homeostasis, and leads to ER stress [17, 78]. Simply defined, ER stress is a disequilibrium between ER load and folding capacity.

Cells cope with ER stress by activating an ER stress signaling cascade called the unfolded protein response (UPR). This activation results in the upregulation of gene expression for molecular chaperones, expands the size of the ER, decreases general protein translation to reduce the ER workload, and degrades abnormal proteins accumulated in the ER [20, 28]. As long as ER stress signaling via the UPR can keep ER stress levels under control, cells can perform their normal functions. However, under certain pathological conditions, including obesity, β-cell exhaustion in type 2 diabetes, and the progression of neurodegeneration, a high level of chronic ER stress persists, leading to cell dysfunction and death [72, 74, 97, 98]. It has been suggested that chronic and high levels of ER stress have a function in β-cell dysfunction and glucose toxicity. In glucose toxicity, insulin secretion by β-cells is impaired in response to stimulation by glucose; the condition is characterized by a sharp decline in insulin gene expression [95, 99]. Numerous studies have shown that impaired β-cell disfunction can be improved by treatment of the hyperglycemia [95, 99], suggesting that identifying the molecular

mechanisms involved in β-cell glucose toxicity may provide new therapeutic targets for diabetes.

Inositol Requiring 1 (IRE1), an ER-resident transmembrane protein kinase, is an upstream component of the UPR and a central regulator of UPR-specific gene expression and apoptosis[47, 53, 89]. Unfolded proteins in the ER lumen are sensed by IRE1, which causes its dimerization, trans-autophosphorylation, and subsequent activation. Activated IRE1 then splices X-box binding protein 1 (XBP-1) mRNA, leading to synthesis of an XBP-1 transcription factor that upregulates specific UPR genes.[47, 48]

If the overload of unfolded proteins in the ER is not resolved, chronic activation of IRE1 leads to activation of the c-Jun N-terminal kinase/stress activated protein kinase (JNK/SAPK) pathway[53]. Chronic ER stress causes IRE1 to bind c-Jun inhibititory kinase and recruit the cytosolic adaptor TRAF2[53]. This results in formation of an IRE1-TRAF2 complex which signals through apoptosis-signaling kinase 1(ASK1) leading to JNK activation. [53, 72, 100, 101] Importantly, IRE1 -/- cells are defective in JNK activation in response to ER stress[53].

Accumulating evidence has implicated JNK signaling in oxidative stress mediated suppression of insulin gene expression (i.e., glucose toxicity)[102]. Transcription of the insulin gene is initiated by the binding of pancreatic and duodenal homeobox factor-1 (PDX-1) to the A elements in the insulin gene promoter [103]. The A elements all share the consensus sequence TAAT, a recognition motif for PDX-1's homeodomain [103, 104]. The decline in insulin gene expression associated with glucose toxicity is accompanied by reduced expression or DNA binding activity of PDX-1 [105-109].

Glucose metabolism has been shown to stimulate PDX-1 activity at the insulin promoter via JNK activation [110]. However, JNK overexpression decreases PDX-1 binding to the insulin promoter, and this correlates with a suppression of both insulin gene expression and secretion. JNK activation by oxidative stress induces the nucleocytoplasmic translocation of PDX-1 thus preventing the DNA binding activity of PDX-1 [111]. Additionally, expression of a dominant negative JNK preserves PDX-1 DNA binding activity in the presence of oxidative stress [102].

Recent studies have shown that ER stress results in suppression of insulin receptor (IR) signaling through hyperactivation of the IRE1-JNK pathway. This leads to JNK induced serine hyperphosphorylation of insulin receptor substrate-1 (IRS-1) [74]. We therefore hypothesized that ER stress mediated hyperactivation of the IRE1-JNK pathway may also have similar effects on the phosphorylation state of PDX-1. In this chapter we show evidence that hyperglycemia induced ER stress hyperactivates the IRE1-JNK pathway resulting in PDX-1 hyperphosphorylation on serine residues. These JNK induced modifications correlate with a reduction of PDX-1 in the nucleus of pancreatic β-cells and may affect PDX-1's ability to activate insulin gene transcription. Our work shows the IRE1α-JNK ER stress signaling pathway is involved in the molecular mechanisms in the reduction of PDX-1 from the nucleus of pancreatic β-cells, however this alone cannot account for the dramatic reduction in insulin mRNA observed during chronic exposure to high glucose levels. Thus our study continues by exploring another mechanism by which ER stress and IRE1α signaling may impact insulin gene expression.

In metazoans, IRE1 activation initiates two separate signaling cascades: an XBP-1-dependent pathway that upregulates ER stress response genes, and an XBP-1-independent pathway involving specific cleavage and subsequent degradation of sets of translating mRNAs on the ER membrane [55]. This response complements other components of the UPR, selectively halting protein synthesis and clearing the translocation machinery when translating mRNAs are overloading the ER and causing ER stress. It has been suggested that this specific mRNA degradation may result from IRE1 focusing on messages that present the most immediate challenge to the translocation and folding machinery[55].

In mice and rats, the two insulin gene transcripts are the most abundantly transcribed mRNAs in β-cells bound for translation through the ER membrane [112]. Global profiling of genes modified by ER stress in β-cells has shown that upon induction of ER stress, insulin 1 and insulin 2 mRNA are quickly degraded, leading to significantly decreased levels of these mRNAs [113]. We have shown in Chapter II that hyperactivation of IRE1 is correlated with a reduction in insulin mRNA expression in pancreatic β-cells [98]. Based on these observations, we hypothesized that when ER folding capacity is overwhelmed, IRE1 initiates endonucleolytic cleavage of mRNA encoding insulin, the major secretory protein in pancreatic β-cells. In this chapter, we suggest that prolonged activation of IRE1 observed upon chronic high glucose exposure may contribute to the reduction in insulin gene expression in two ways; IRE1α-mediated insulin mRNA degradation, and IRE1α-JNK-mediated PDX-1 inactivation. These effects may act synergistically to decrease insulin mRNA. What follows is our characterization

of these two effects elicited by IRE1 signaling in pancreatic β-cells. Here we report that IRE1α hyperactivation has a function in insulin mRNA reduction under chronic high-glucose conditions and implicate ER stress in the molecular mechanisms of glucose toxicity.

Materials and Methods

Cell culture and transfection of small interfering RNA and plasmid DNA

Rat insulinoma cells, INS-1 832/13 cells, were a gift from Dr. Christopher Newgard (Duke University Medical Center). INS-1 832/13, and mouse islets were cultured in RPMI 1640 supplemented with 10% FBS. COS7 cells were cultured in DMEM supplemented with 10% FBS and transfected using the FuGene Transfection Reagent (Roche, Basel, Switzerland). Cell Line NucleofectorTM Kit V with the Nucleofector Device (Amaxa Biosystems, Gaithersburg, MD) was used to transfect small interfering RNA (siRNA) for IRE1α into INS1 832/13 cells. At QIAGEN (Valencia, CA) siRNAs for rat IRE1α were designed and synthesized: for rat IRE1α, AAGGCGATGATCTCAGACTTT. Nucleofection was also used to transfect INS1 832/13 cells with pcDNA3.0 and DN-Traf2-Flag in pcDNA3.0.

Separation of nuclear and cytoplasmic cell lysates and immunoblotting

Lysates were extracted using NE-PER® Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology, Rockford, IL) supplemented with protease inhibitors. Lysates were normalized for total protein (20 µg per lane), separated using 4%-20% linear gradient SDS-PAGE (Bio Rad, Hercules, CA), and transferred to a nitrocellulose membrane. After blocking for 1h at room temperature in TBS-T with 5% nonfat dry milk, membranes were incubated at 4°C overnight in TBS-T with 5% nonfat dry milk containing a 1:1000 dilution of antibody and washed 3 times with TBS-T. The

membranes were then incubated for 1 h at room temperature in TBS-T with 5% nonfat dry milk containing a 1:3000 dilution of anti-rabbit IgG antibody (Bio Rad) coupled to horseradish peroxidase, followed by washing 3 times with TBS-T. Immunoreactive bands were visualized by incubation with Pierce ECL and exposed to x-ray film. For total protein lysate analysis, cells were lysed in ice-cold M-PER buffer (PIERCE, Rockford, IL) containing protease inhibitors for 15 min on ice. The lysates were then cleared by centrifuging the cells at 13,000 x g for 15 min at 4°C. Lysates were normalized for total protein (10 µg per lane), separated using 4%-20% linear gradient SDS-PAGE (Bio Rad, Hercules, CA), and electroblotted. Anti-phospho IRE1α antibody was generated from bulk antiserum by affinity purification followed by adsorption against the nonphospho analog column peptide [98]. Total IRE1α was measured with anti-total-IRE1α antibody NY200 [25]. Anti-actin antibody and Anti-Flag antibody were purchased from Sigma (St. Louis, MO). Anti-PDX-1 and anti-phospho-JNK antibodies were purchased from Upstate/Cell Signaling (Lake Placid, NY) XBP-1 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA)

Generation of IRE1α heterozygous mice

Mice heterozygous for the *Ire1α* (*Ire1α*$^{+/-}$) gene [53] were backcrossed with 129SvEv mice more than ten generations to obtain an essentially congenic 129SvEv genetic background.

Real-time polymerase chain reaction

Total RNA was isolated from the cells by using RNeasy Mini Kit (Qiagen), then reverse-transcribed using 1 μg of total RNA from cells with Oligo-dT primer. For the thermal cycle reaction, the iQ5 system (BioRad) was used at 95°C for 10 min, 40 cycles at 95°C for 10 sec, and at 55°C for 30 sec. The relative amount of each transcript was calculated by a standard curve of cycle thresholds for serial dilutions of cDNA sample and normalized to the amount of actin. PCR was done in triplicate for each sample. The following sets of primers and Power SYBR Green PCR Master Mix (Applied Biosystems) were used for real-time PCR: for mouse actin, GCAAGTGCTTCTAGGCGGAC and AAGAAAGGGTGTAAAACGCAGC; for mouse insulin1, GAAGTGGAGGACCCACAAGTG and CTGAAGGTCCCCGGGGCT; for mouse insulin2, TGCTGATGCCCTGGCCTGCTCT and CTGGTCCCACATATG CACATGCA; for mouse CHOP, CCACCACACCTGAAAGCAGAA and AGGTGAAAGGCAGGGACTCA; for mouse total XBP-1, TGGCCGGGTCTGCTGAGTCCG and GTCCATGGGAAGATGTTCTGG; for mouse spliced XBP-1, CTGAGTCCGAATCAGGTGCAG (original CAG sequence was mutated to AAT to reduce the background signal from unspliced XBP-1) and GTCCATGGGAAGATGTTCTGG; for rat actin, GCAAATGCTTCTAGGCGGAC and AAGAAAGGGTGTAAAACGCAGC; for rat Glut2, GTGTGAGGATGAGCTGCCTAAA and TTCGAGTTAAGAGGGAGCGC; for rat insulin 1, GTCCTCTGGGAGCCCAAG and ACAGAGCCTCCACCAGG; for rat insulin 2, ATCCTCTGGGAGCCCCGC and AGAGAGCTTCCACCAAG; for rat spliced

XBP-1, CTGAGTCCGAATCAGGTGCAG (original CAG sequence was mutated to AAT to reduce the background signal from unspliced XBP-1) and ATCCATGGGAAGATGTTCTGG.

mRNA Degradation

Cellular mRNA transcription was attenuated by treating cells with 100 µg/mL actinomycin D (Sigma A-4262) for 1 hr followed by treatment with 1µM thapsigargin for different time periods. Total RNA was isolated from the cells using the RNeasy Mini Kit (Qiagen). Reverse-transcribed RNA was subjected to real-time PCR quantitation to measure levels of insulin gene transcripts. The relative amount of each transcript was calculated by a standard curve of cycle thresholds for serial dilutions of cDNA sample and normalized to the amount of actin. Time point zero for each condition was standardized to 1 and the subsequent rate of degradation of mRNA was measured.

Results

Early inquiries into the effects of long term exposure to high glucose included a study that demonstrated that glucose can decrease insulin gene expression in INS-1 cells, a cell line able to secrete insulin in response to physiologic glucose concentrations [107]. Surprisingly, the researchers observed that the glucose-induced decrease in insulin gene expression occurred after only 48 hours of exposure to high glucose concentrations. However, they did observe the same diminished binding activity of PDX-1 to the insulin gene promoter seen in cell lines treated with glucose for weeks or months. Their results suggest that similar molecular mechanisms may be at work in the glucose desensitization of the insulin gene in the INS-1 cell line. INS-1 cells are therefore an excellent *in vitro* model to study glucose toxicity and thus are our cells of choice in the following study.

ER stress activates IRE1α and correlates with a reduction of PDX-1 from the nucleus of pancreatic β-cells

To study the role of ER stress in PDX-1 nuclear localization, we first treated INS-1 832/13 cells for 3 hours with increasing concentrations of glucose, as well as physiological levels of glucose with experimental ER stress inducing drugs tunicamycin or thapsigargin (Figure 3.1A). Nuclear PDX-1 was lowest in 2.5 mM glucose treated cells, and increased correspondingly as glucose concentration increased. However, inducing ER stress by treatment with both tunicamycin and thapsigargin reduced nuclear PDX-1, with thapsigargin having the most dramatic effect. As we have previously

reported, tunicamycin was the strongest inducer of XBP-1 splicing, while thapsigargin was the strongest activator of JNK phosphorylation.

To confirm that thapsigargin was activating ER stress signaling pathways, we treated INS-1 832/13 cells with low glucose, high glucose, or thapsigargin for 3 hours and measured IRE1α phosphorylation by immunoblot (Figure 3.1B). We observed IRE1α activation by both high glucose and thapsigargin, however, strong JNK phosphorylation was seen only by treatment with thapsigargin. Because chronic treatment of β-cells with high glucose correlates with very strong phosphorylation of IRE1α , and because JNK has been solidly implicated in reduction of PDX-1 from the nucleus, we next treated INS-1 832/13 cells with low or high glucose for 3 days. We observed that treatment with 16.7 mM glucose for 3 days strongly activated both IRE1α and JNK, and correlated with a reduction in nuclear PDX-1 (Figure 3.1C). We therefore hypothesized that activation of the IRE1α-JNK ER stress signaling pathway by chronic high glucose treatment is similar to the transient activation of this pathway in only 3 hours by the drug thapsigargin. That is, both chronic high glucose and transient treatment with thapsigargin cause strong activation of IRE1α and its downstream effector; JNK.

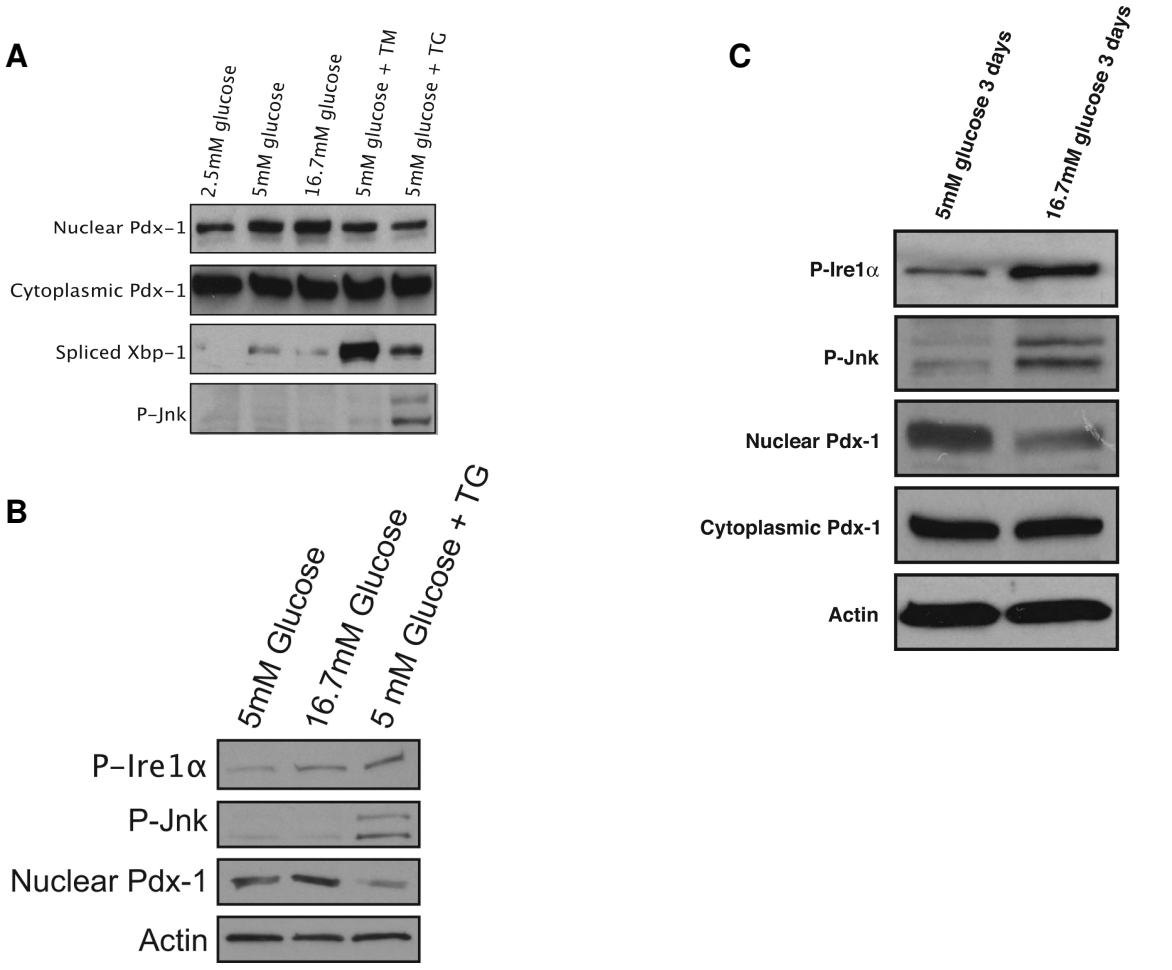

Figure 3.1. ER stress activates IRE1α and correlates with a reduction of PDX-1 from the nucleus of pancreatic β-cells

A) INS-1 832/13 cells were pretreated with 5 mM glucose for 20 hours, and then with 2.5 mM glucose for 1 hour. The cells were then stimulated with KRB buffer containing 2.5 mM, 5 mM, or 16.7 mM glucose or 5 mM glucose plus tunicamycin (2.5 µg/mL) or thapsigargin (1 µM) for 3 hours. Cytoplasmic and nuclear fractions were collected and cellular expression levels of cytoplasmic and nuclear PDX-1, spliced XBP-1, phosphorylated JNK and actin were measured by immunoblot.

B) INS-1 832/13 cells were pretreated with 5 mM glucose for 20 hours, and then with 2.5 mM glucose for 1 hour. The cells were then stimulated with KRB buffer containing 5 mM or 16.7 mM glucose or 5 mM glucose and thapsigargin (1 µM) for 3 hours. Cytoplasmic and nuclear fractions were collected and cellular expression levels of phosphorylated IRE1α, cytoplasmic and nuclear PDX-1, and actin were measured by immunoblot.

C) INS-1 832/13 cells were incubated in 5 mM glucose RPMI or 16.7 mM glucose RPMI for 72 hours. Cytoplasmic and nuclear fractions were collected and expression levels of phospho-IRE1α, phospho-JNK, cytoplasmic and nuclear PDX-1, and actin were measured by immunoblot.

Inhibition of the IRE1α-JNK signaling pathway partially restores PDX-1 protein to the nucleus of pancreatic β-cells

To test the involvement of the IRE1α-JNK signaling pathway in PDX-1 cellular localization directly, we inhibited the expression of IRE1α in INS-1 832/13 cells using siRNA specific for IRE1α (Figure 3.2A). After a 20 hour treatment with either IRE1α siRNA or control siRNA, we treated the cells with low glucose, high glucose, or thapsigargin for 3 hours and measured total IRE1α, JNK phosphorylation and PDX-1. The suppression of IRE1α expression decreased the amount of JNK phosphorylation and restored the amount of nuclear PDX-1 in the cells, as compared to treatment with control siRNA.

It has been previously shown the IRE1α activates JNK through the cytosolic adapter protein TRAF2. To this end, we examined the dominant negative effect of a mutant TRAF2 protein on PDX-1 cellular localization (Figure 3.2B). After transfection with either DN-TRAF2 or pcDNA3.0 control plasmid, cells were treated with low glucose, high glucose, or thapsigargin for 3 hours and again total IRE1α, JNK phosphorylation and PDX-1were measured. The suppression of TRAF2 signaling both reduced JNK phosphorylation upon ER stress and partially preserved PDX-1 protein in the nucleus. Taken together, these results suggest that the IRE1α-JNK ER stress signaling pathway is involved in the molecular mechanisms in the reduction of PDX-1 from the nucleus of pancreatic β-cells.

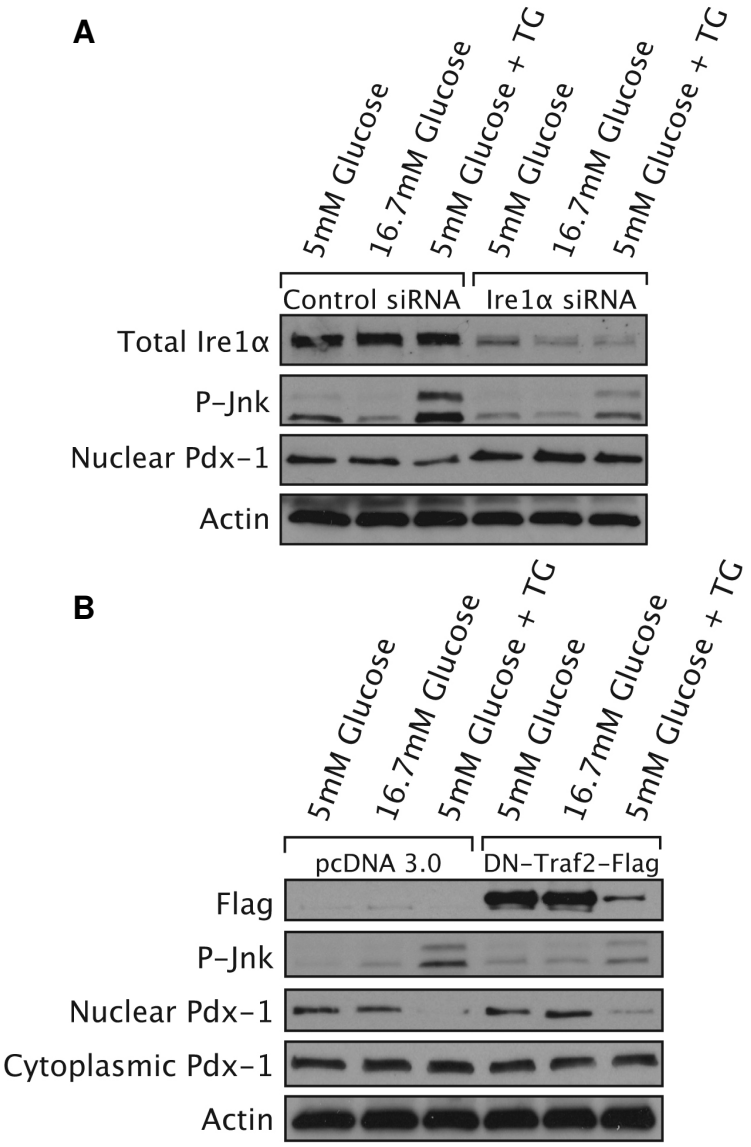

Figure 3.2. Inhibition of the IRE1α-JNK signaling pathway restores PDX-1 protein to the nucleus of pancreatic β-cells

A) INS-1 832/13 cells were transfected with siRNA specific for rat IRE1α (final concentration 20 nM) or control siRNA and incubated in 5mM glucose. 20 hours post-transfection, cells were incubated in 2.5 mM glucose for 1 hour. The cells were then stimulated with KRB buffer containing 5 mM or 16.7 mM glucose or 5mM glucose and thapsigargin (1 μM) for 3 hours. Cytoplasmic and nuclear fractions were collected and cellular expression levels of total IRE1α, cytoplasmic and nuclear PDX-1, phospho-JNK, and actin were measured by immunoblot.

B) INS-1 832/13 cells were transfected with either a dominant negative form of TRAF2 tagged with Flag or with pcDNA3.0 plasmid as a control and incubated in 5 mM glucose for 20 hours. Cells were then pretreated with 2.5 mM glucose for 1 hour and stimulated with KRB buffer containing 5 mM or 16.7 mM glucose or 5 mM glucose and thapsigargin (1 μM) for 3 hours. Cytoplasmic and nuclear fractions were collected and cellular expression levels of Flag, cytoplasmic and nuclear PDX-1, phospho-JNK, and actin were measured by immunoblot.

Chronic high glucose causes intense activation of the IRE1-JNK pathway and strong serine phosphorylation of PDX-1 by JNK.

Hyperactivation of the IRE1α-JNK signaling pathway in liver cells leads to increased serine phosphorylation of IRS-1 by JNK [74]. We questioned whether this could be an explanation for PDX-1 reduction from the nucleus upon ER stress in pancreatic β-cells. We treated INS-1 832/13 cells with low glucose, high glucose, or thapsigargin for 3 hours or treated INS-1 832/13 cells with 5 mM or 16.7 mM glucose for 3 days followed by immunoprecipitation with anti-PDX-1 antibody (Figure 3.3 A and B). We then immunoblotted with antibody specific for phosphorylation on serine residues. We observed strong serine phosphorylation in the cells treated with 16.7 mM glucose for 3 days. However, we did not observe this same phosphorylation of serine for the 3 hour thapsigargin treated cells. This result suggests that the effects on the phosphorylation of PDX-1 by chronic high glucose treatment cannot be mimicked with transient treatment with thapsigargin in only 3 hours. Although both chronic high glucose and transient thapsigargin treatment cause ER stress and activation of the IRE1α-JNK signaling pathway, the results indicate that the mechanisms whereby PDX-1 is reduced from the nucleus differ. Nevertheless, our results suggest that under chronic hyperglycemia, IRE1α-JNK signaling may decrease insulin gene transcription via phosphorylation of PDX-1. PDX-1 phosphorylation has been reported to cause its export from the nucleus as well as to interfere with PDX-1 binding to the insulin promoter [114, 115]. Our data suggest a role for ER stress and IRE1α activation in this process.

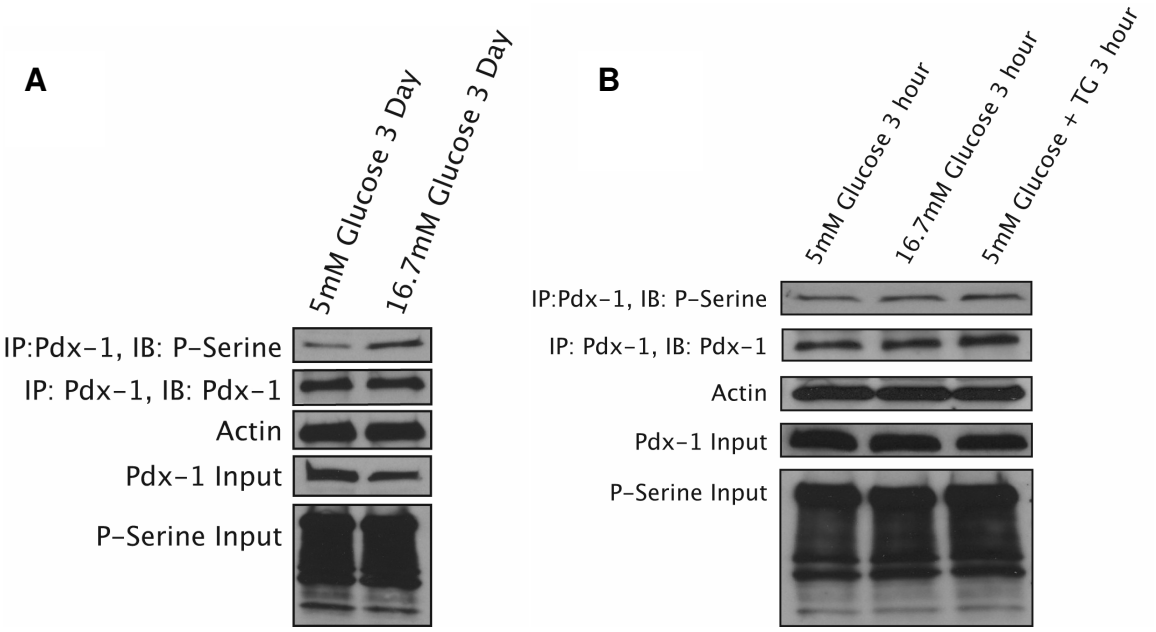

Figure 3.3. Chronic high glucose, but not experimental ER stress inducers, cause intense activation of the IRE1-JNK pathway and strong serine phosphorylation of PDX-1 by JNK.
A) INS-1 832/13 cells were incubated in 5 mM glucose RPMI or 16.7 mM glucose RPMI for 72 hours. PDX-1 was immunoprecipitated (IP) using total cell lysates. IP products were immunoblotted (IB) with anti-PDX-1 antibody and anti-phospho-serine antibody. Input protein was also run as a control and immunoblotted with anti-phospho-serine antibody.

B) INS-1 832/13 cells were pretreated with 5 mM glucose for 20 hours, and then with 2.5 mM glucose for 1 hour. The cells were then stimulated with KRB buffer containing 5 mM or 16.7 mM glucose or 5 mM glucose and thapsigargin (1 μM) for 3 hours. PDX-1 was immunoprecipitated (IP) using total cell lysates. IP products were immunoblotted with anti-PDX-1 antibody and anti-phospho-serine antibody. Input protein was also run as a control and immunoblotted (IB) with anti-phospho-serine antibody.

Chronic high glucose causes ER stress and reduces insulin gene expression in pancreatic β-cells

Our work has shown that the IRE1α ER stress signaling pathway is involved in the molecular mechanisms of the reduction of PDX-1 from the nucleus of pancreatic β-cells. However, this alone cannot account for the dramatic reduction in insulin mRNA observed during chronic exposure to high glucose levels. Additionally, treatment with thapsigargin has been shown to decrease insulin mRNA without affecting the insulin gene promoter [113], suggesting that a mechanism independent of PDX-1 inactivation is responsible for the reduction of insulin mRNA. Thus, our study continued by exploring another mechanism by which ER stress and IRE1α signaling may impact insulin gene expression. To investigate the effect of chronic high-glucose treatment on expression levels of insulin and ER stress response genes, we treated INS-1 832/13 cells and primary mouse islets with increasing concentrations of glucose for 24 and 72 hours, then measured expression levels of insulin 1 and insulin 2 gene expression, as well as expression of several well-known markers of ER stress.

After 24 hours, both insulin 1 and insulin 2 gene expression increased with increasing glucose. Expression levels of spliced Xbp-1 and Ero1α, ER stress markers, also increased with glucose concentration. The expression of Chop, an ER stress marker of apoptosis remained the same, suggesting that chronic glucose treatment causes mild ER stress, but not cell death (Figure 3.4A). Seventy-two hour treatment with high glucose caused a dramatic reduction in both Insulin 1 and Insulin 2 gene expression, an indication of glucose toxicity (Figure 3.4B). We also observed a decrease in Xbp-1 splicing and

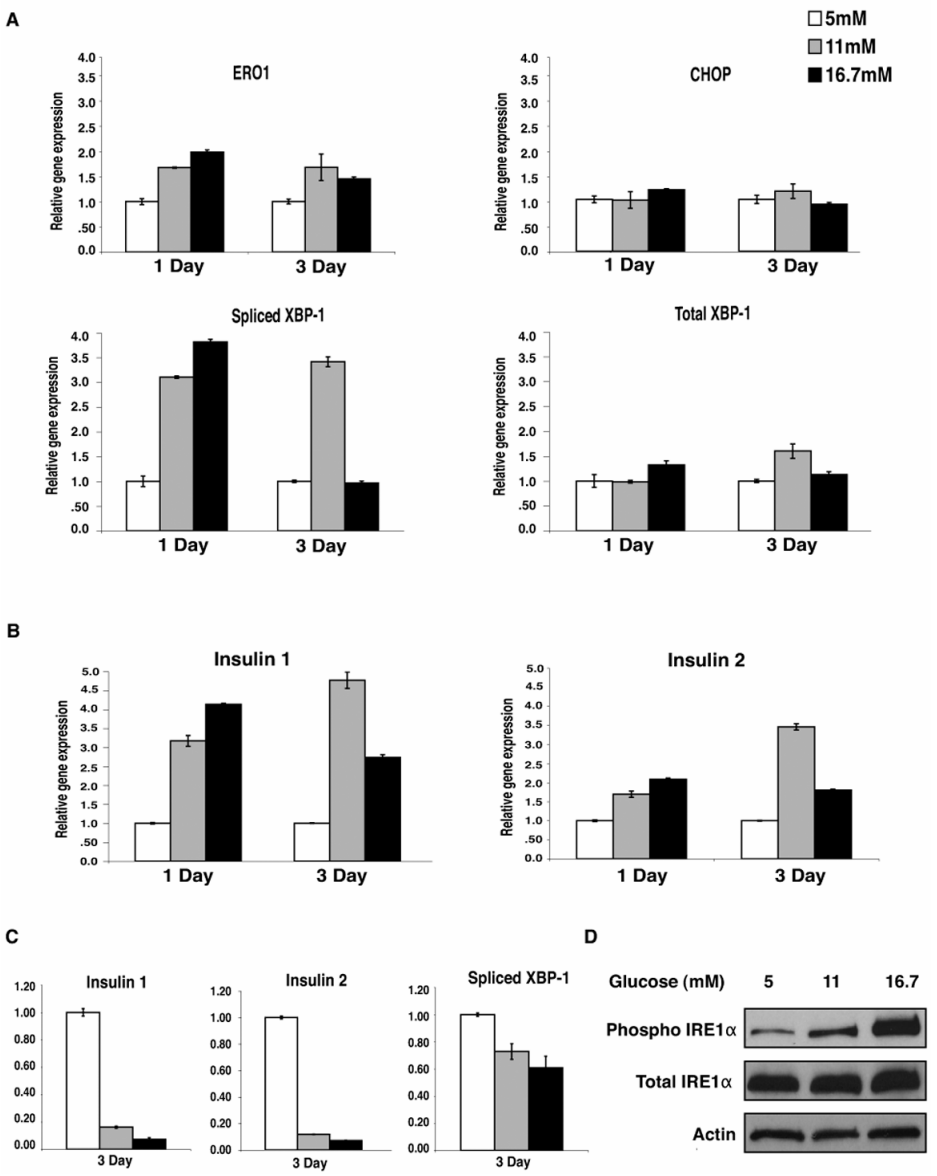

Figure 3.4. Chronic high-glucose treatment causes ER stress in islets and insulinoma cells, resulting in a reduction in insulin gene expression.

A) Islets pooled from 6 mice were treated with 5 mM, 11 mM, or 16.7 mM glucose for 24 or 72 hours. Expression levels of Ero1α, Chop, spliced and total Xbp-1 were measured by real- time PCR (n = 2).

B) Islets pooled from 6 mice were treated with 5 mM, 11 mM, or 16.7 mM glucose for 24 or 72 hours. Expression levels of insulin 1 and insulin 2 were measured by real time PCR (n = 2).

C) INS-1 832/13 cells were pretreated for 12 hours with 5 mM glucose, then treated with 5 mM, 11 mM, or 16.7 mM glucose for 72 hours. Expression levels of insulin 1, insulin 2, and spliced Xbp-1 were measured by real time PCR (n = 3; values are mean±SEM).

D) INS-1 832/13 cells were pretreated for 12 hours with 5 mM glucose, then treated with 5 mM, 11 mM, or 16.7 mM glucose for 72 hours. Total IRE1α, phosphorylated IRE1α, and actin were measured by immunoblot.

other ER stress markers in the islets after 72 hour treatment with 16.7 mM glucose (Figure 3.4A), and with both 11 mM and 16.7 mM in INS-1 832/13 cells (Figure 3.4C). This decrease in Xbp-1 splicing directly correlated with the decrease in insulin 1 and insulin 2 gene expression. However, we observed that phosphorylation of Ire1α increased as glucose concentration increased, and was much stronger after 72 hours of treatment, despite the reduction in XBP-1 splicing (Figure 3.4D).

IRE1α overexpression correlates with reduced insulin mRNA

Our results suggested that chronic high glucose activates an Xbp-1 independent Ire1α signaling cascade. The strong activation of Ire1α, combined with the loss of Xbp-1 splicing during chronic high-glucose treatment, led us to hypothesize that IRE1α itself may have a direct function in the degradation of insulin mRNA. To test this hypothesis, we transfected COS-7 cells with mouse insulin 2 expression plasmid, then transfected wild-type human IRE1α expression plasmid or a kinase/endoribonuclease inactive dominant-negative mutant K599A IRE1α expression plasmid into these cells, then measured insulin gene expression (Figure 3.5A). We observed a reduction in insulin 2 mRNA in cells overexpressing wild-type IRE1α, but not in cells overexpressing mutant K599A IRE1α or the pcDNA3 vector control.

We also transfected either wild-type human IRE1α or K599A IRE1α expression plasmids into INS-1 832/13 cells and measured endogenous insulin 1 and insulin 2 mRNA expression (Figure 3.5B). We found downregulation of both insulin 1 and insulin 2 gene expression only in cells overexpressing wild-type IRE1α.

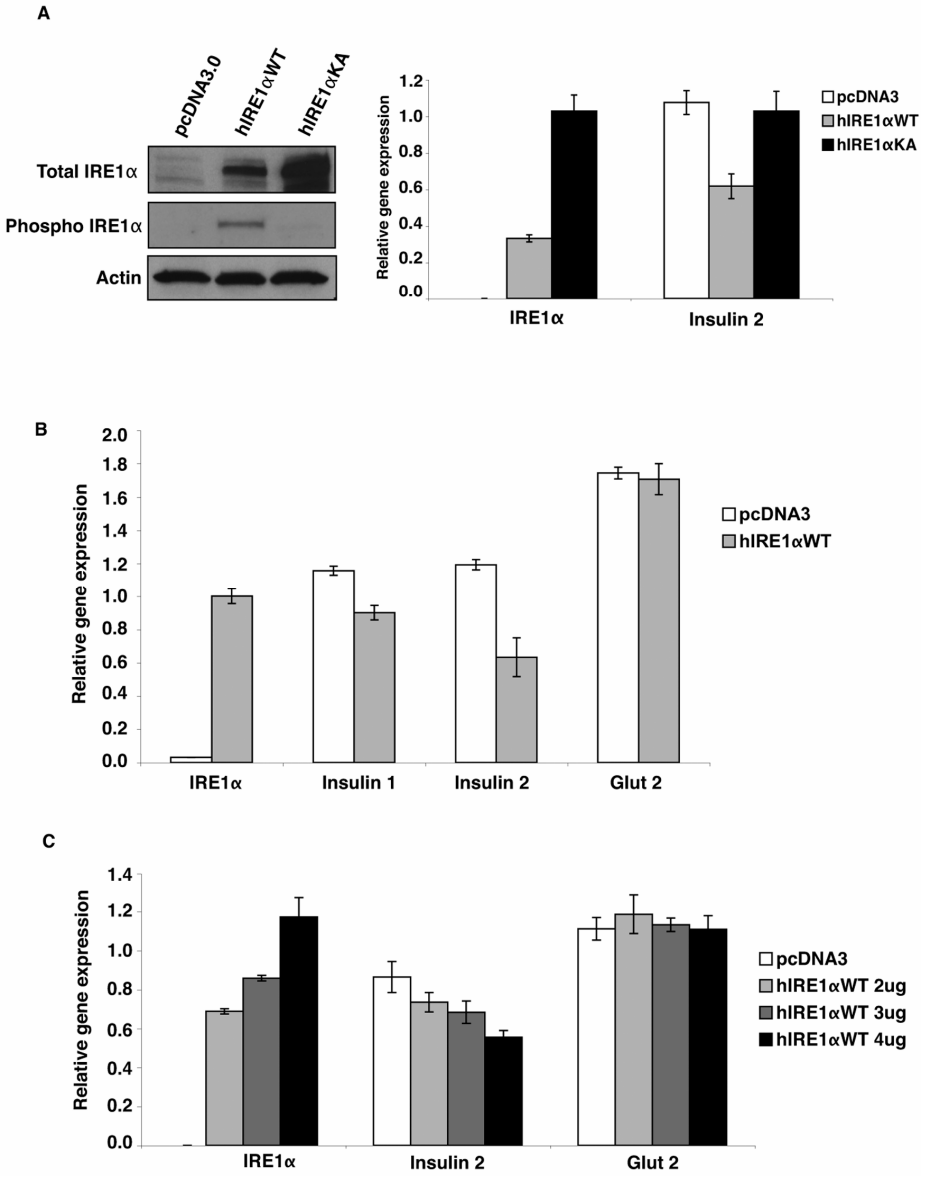

Figure 3.5. Overexpression of IRE1α correlates with reduced Insulin mRNA in cultured cells.
A) COS-7 cells were transfected with mouse insulin 2 and cultured for 24 hours. Cells were then split into 3 plates and transfected again with wild-type human Ire1α; IRE1α WT, a kinase/endoribonuclease inactive mutant human Ire1α; IRE1α KA; or pcDNA3 control. They were then cultured for 24 hours. Protein and RNA were collected from the same plates. Total IRE1α, phosphorylated IRE1α, and actin were measured by immunoblot. Expression levels of human IRE1α and mouse Insulin 2 were measured by real time PCR (n = 3; values are mean±SEM).

B) INS-1 832/13 cells were transfected with human IRE1α WT or pcDNA3 control and cultured for 24 hours. Expression levels of human IRE1α, endogenous rat insulin 1, insulin 2, and glucose transporter 2 (glut 2) were measured by real time PCR (n = 3; values are mean±SEM).

C) INS-1 832/13 cells were transfected with either pcDNA3 control or increasing concentrations of human IRE1α WT and cultured for 24 hours. Expression levels of human IRE1α, endogenous rat Insulin 1, Insulin 2, and glucose transporter 2 (glut 2) were measured by real-time PCR (n = 3; values are mean±SEM).

To study the correlation between IRE1α expression levels and insulin gene expression, we expressed increasing amounts of wild-type IRE1α in INS-1 832/13 cells and measured expression levels of insulin. The results indicated a dose-dependent response to the amount of IRE1α expressed and the fold reduction in endogenous insulin mRNA (Figure 3.5C). Taken together, these data show a firm correlation between strong activation of IRE1α and the reduction of insulin mRNA independent of glucose concentration.

Inhibition of IRE1α activation by a dominant negative mutant blocks both high glucose induced and thapsigargin induced insulin mRNA degradation

To test the hypothesis that IRE1α is directly involved in the reduction of insulin mRNA upon exposure to chronic high glucose, we generated INS-1 832/13 cell lines expressing the dominant-negative mutant K599A IRE1α, using a lentivirus-based doxycycline-mediated induction system. We induced expression of K599A IRE1α or empty vector control in INS-1 832/13 cells, then challenged them with increasing concentrations of glucose for 72 hours (Figure 3.6A). As compared to the INS-1 832/13 control cells, cells stably expressing the dominant-negative mutant K599A IRE1α resisted the high glucose-induced reduction in insulin mRNA and had higher gene expression for both insulin 1 and insulin 2. Thapsigargin, a chemical ER stress inducer, causes a significant decrease in the transcript levels of insulin 1 and insulin 2, while insulin promoter activity remains unaffected [113]. To determine whether IRE1α is responsible for this chemically induced ER stress decrease in insulin mRNA expression, we used actinomycin D to attenuate mRNA transcription then challenged the cells with

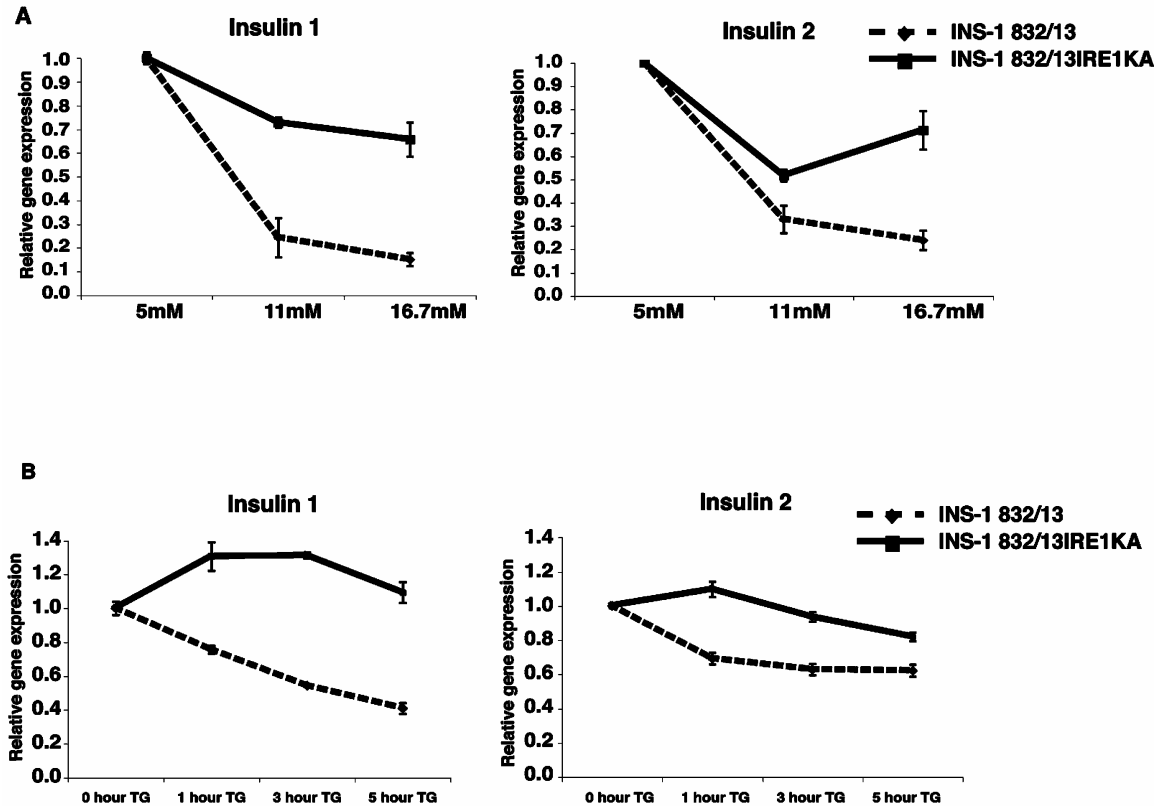

Figure 3.6. Cells expressing mutant IRE1α resist both chemical- and glucose-induced Insulin mRNA degradation.

A) pTetRINS-1832/13 cells (control) and pTetRINS-1832/13IRE1αKA cells (stably expressing tetracycline-responsive K599A mutant IRE1α) were treated with doxycycline for 24 hours with 5 mM glucose to induce mutant IRE1α. Cells were then treated with 5 mM, 11 mM, or 16.7 mM glucose with doxycycline for 72 hours. Degradation of mRNA was assessed by measuring the expression of insulin 1 and insulin 2 by real-time PCR (n = 3; values are mean±SEM).

B) pTetRINS-1 832/13 cells (control) and pTetRINS-1832/13IRE1αKA cells (stably expressing tetracycline-responsive K599A mutant IRE1α) were treated with doxycycline for 24 hours to induce mutant IRE1α. mRNA transcription was attenuated by treating cells with 100 µg/mL actinomycin D for 1 hour. To induce degradation of insulin mRNA, 1 µM thapsigargin was added to the medium for 0, 1, 3 and 5 hours. mRNA degradation was assessed by measuring expression of insulin 1 and insulin 2 by real-time PCR (n = 3; values are mean±SEM).

thapsigargin to induce insulin 1 and insulin 2 mRNA degradation. Cells expressing the dominant-negative mutant K599A IRE1α resisted the thapsigargin-induced decrease in both insulin 1 and insulin 2 gene transcripts observed in control cells (Figure 3.6B).

Islets from mice heterozygous for IRE1α are resistant to high-glucose-mediated reduction in insulin mRNA expression

We observed that cells in which strong activation of IRE1α was partially blocked by use of a mutant form of IRE1α were more resistant than control cells to the glucotoxic effects of chronic high glucose on insulin gene expression. We then tested whether islets from mice heterozygous for IRE1α are also more resistant to chronic high glucose. We treated islets from mice that were heterozygous for IRE1α or islets from their wild-type littermates with 16.7 mM glucose for 72 hours, then measured insulin 1 and insulin 2 gene expression and Xbp-1 splicing (Fig. 3.7). Heterozygous mouse islets were more resistant to the glucotoxic effects of chronic high glucose exposure, in that they had higher insulin 1 and insulin 2 gene expression than did their wild-type littermate controls. However, splicing of Xbp-1 was lower in the IRE1α heterozygotes, suggesting that these mice were also more resistant to ER stress induced by chronic high glucose. These results suggest that IRE1α acts directly in the reduction of insulin mRNA under chronic ER stress conditions, and that blocking the activation of IRE1α under these conditions can protect cells from negative effects.

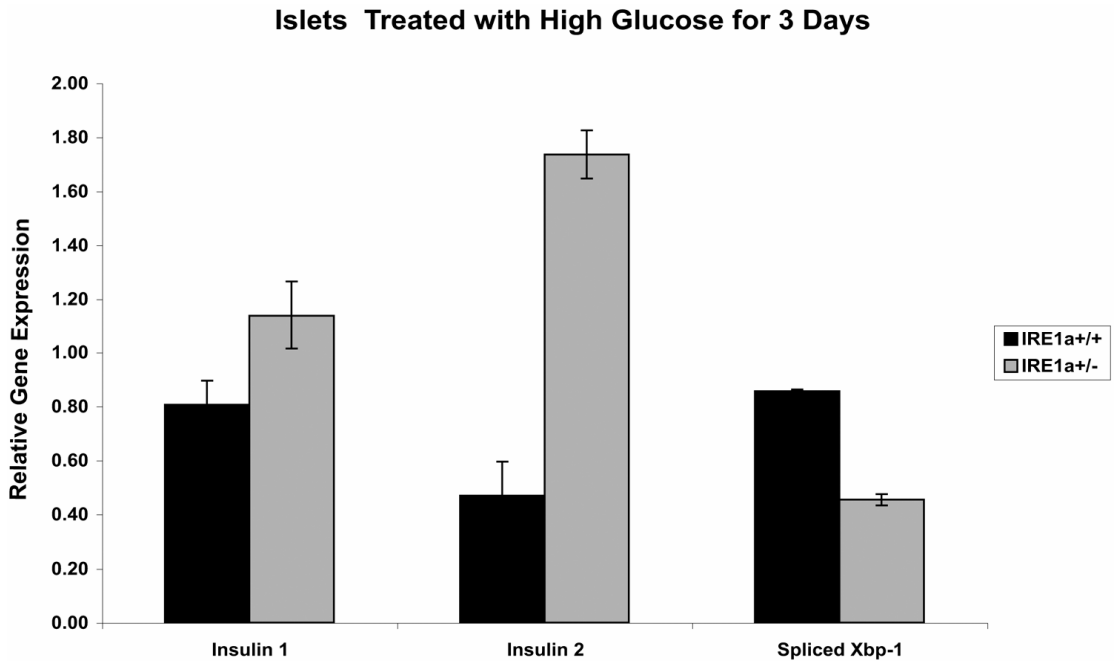

Figure 3.7. IRE1α heterozygous islets resist the negative effects of chronic high glucose.

IRE1α WT or IRE1α heterozygous mouse islets were treated with 16.7 mM glucose for 72 hours. Expression of Insulin 1, Insulin 2, and spliced XBP-1 were measured by quantitative real-time PCR.

Discussion

Our results demonstrate a genetic and biochemical linkage between ER stress signaling and reduction in insulin mRNA expression in pancreatic β-cells under chronic high glucose conditions. We propose that IRE1α hyperactivation by chronic high glucose results in selective degradation of insulin mRNA, leading to glucose toxicity. It has been shown that insulin mRNA degrades rapidly under ER stress conditions in pancreatic β-cells[113]. However, the precise mechanism whereby IRE1-mediated insulin mRNA degradation occurs is unclear. The reduction of insulin mRNA under ER stress conditions may be initiated by direct endonucleolytic cleavage by the nuclease domain of IRE1, ultimately leading to degradation of the insulin message. Alternatively, IRE1 may function in the activation or recruitment of additional ribonucleases that can degrade insulin messages. It is also possible that IRE1 signaling may somehow initiate insulin gene-specific transcriptional stalling. Regardless of the precise mechanism, our data show that IRE1α, a central component of ER stress signaling, has an essential function in the reduction of insulin mRNA.

Chronically high levels of glucose also cause oxidative stress, leading to activation of c-Jun N-terminal protein kinase (JNK). This JNK activation suppresses PDX-1 binding to the insulin promoter and reduces insulin gene expression [24]. We have shown previously that in mammalian cells, ER stress signaling activates JNK through IRE1 [53]. Our data suggest that chronic high glucose causes ER stress induced activation of IRE1α-JNK signaling. Thus, hyperactivation of IRE1α by chronic high

glucose may also suppress insulin gene expression partially through JNK-mediated PDX-1 inactivation leading to reduced transcription of the insulin gene.

Numerous studies have implicated PDX-1 and MafA, two transcription factors that are important for insulin gene transcription, in the defective insulin gene expression in β-cells caused by chronic exposure to supraphysiologic concentrations of glucose[105, 106, 116-119]. Chronic exposure of β-cells to excess glucose decreases PDX-1 gene expression and MafA protein expression, leading to the suppression of insulin gene expression. Our current results suggest that mRNA degradation is an additional contributor to the reduction in insulin gene expression observed upon chronic exposure to high glucose. All of these effects may act synergistically to decrease insulin mRNA.

Our work demonstrates that two distinct activities are elicited by high-glucose-induced activation of IRE1α in pancreatic β-cells. IRE1α can be activated in β-cells by overexpressing insulin; and moreover, the level of activation positively correlates with the amount of insulin (Lipson and Urano, unpublished observations). We therefore believe that exposure of β-cells to high glucose levels causes ER stress due to an increased load of insulin translation into the ER. In earlier studies, we found that IRE1α signaling, activated by acute exposure to high glucose, enhances proinsulin biosynthesis [98]. In contrast, chronic exposure of β-cells to high glucose causes hyperactivation of IRE1α, leading to the degradation of insulin mRNA. Thus, we propose that the duration of exposure to high glucose, and therefore, the relative load of translocating insulin, are the critical determinants of the activity of IRE1α.

High glucose exposure causes ER stress and upregulation of ER folding machinery. Insulin mRNA expression increases, but only to a point. This point may represent the time when the burden that the translocating insulin is placing on the ER exceeds the ER processing capacity. In this scenario, the "classical" solution would be to activate Xbp-1 splicing and synthesize more ER folding machinery. However, this upregulation of ER stress-response proteins may add to the burden of the already overloaded ER. The rapid degradation of insulin mRNA could provide immediate relief to the ER and free the translocation machinery. Thus, this mechanism may be an essential element in the adaptation of β-cells to chronic hyperglycemia. This rapid mRNA degradation, combined with the IRE1α-JNK induced inactivation of PDX-1, may act synergistically to reduce insulin gene expression.

Chronic ER stress has recently been defined as any persistent (on the order of days to years) stress that requires long term adjustments in cellular function [20]. For cells to survive under a chronic ER stress condition like prolonged hyperglycemia, they must have a mechanism by which ER stress can be continuously tolerated. A small number of cells may die, but the majority of cells must survive and adapt to the stressful stimulus, which, in this case, is chronic exposure to high glucose levels. This adaptation may be crucial for the maintenance of β-cell homeostasis and may, in part, explain why the β-cells of Type 2 diabetic patients with chronic hyperglycemia stop producing insulin without simply undergoing apoptosis.

The IRE1-mediated mRNA decay pathway may not, however, be limited to a stress-response function. In other types of secretory cells, this mechanism of selective

degradation of mRNAs by IRE1 may be an effective way to quickly control levels of secretory proteins. In addition to transducing the ER stress response, secretory cells may also activate IRE1 in response to various cellular stimuli, allowing adaptation to rapidly changing physiological conditions. In β-cells, it remains to be seen whether these reductive effects of IRE1α on insulin mRNA are actually important for insulin protein biogenesis, which is regulated at many levels. Modulation of levels of its message is certainly one place for regulation. If the load of insulin folding and processing is exceeding the capacity of the ER, then rapid reduction of insulin mRNA would preserve ER homeostasis and help ensure that the insulin already inside the ER can be properly folded and secreted. This mechanism may also be involved in suppression of the autoimmune response by reducing the amounts of misfolded insulin, which could be a source of "neo-autoantigens."

Based on recent studies and the results reported here, we suggest that in β-cells IRE1α selectively degrades insulin, the most prevalent ER-targeted mRNA, under adverse conditions. This may be part of a protective adaptation that β-cells have uniquely acquired to protect themselves from death caused by the chronic and high workload placed on the ER under prolonged hyperglycemic and/or hyperinsulinemic conditions. This, combined with subsequent upregulation of ER stress response genes, may function in support of β-cell survival under extreme stress conditions such as chronic hyperglycemia.

CHAPTER IV

DISCUSSION AND PERSPECTIVES

Diabetes is quickly reaching epidemic proportions worldwide, and its heterogeneous nature has made determining underlying causes somewhat difficult. When we began our studies, there existed some genetic evidence implicating ER stress and defective ER stress signaling in the development of rare forms of experimental and clinical diabetes. This evidence suggested that activation of certain ER stress signaling pathways may be critical to the normal function of insulin-secreting β-cells, and that β-cells may also be especially sensitive to the adverse effects of perturbed ER function. The work presented in this dissertation was undertaken to reveal physiological, as well as pathological roles, of ER stress signaling in pancreatic β-cells. This information furthers our understanding of the normal function of pancreatic β-cells and also sheds light on processes involved in insulin biosynthesis and β-cell homeostasis.

In Chapter II we presented evidence that activation of IRE1α by high glucose treatment has a beneficial effect in β-cells and aids in the proper folding of proinsulin inside the ER. Glucose exposure stimulates insulin biosynthesis[10]. Here we present a model whereby glucose-mediated stimulation of proinsulin biosynthesis promotes some ER stress because it imposes a load on the protein folding and processing machinery of the ER. This results in activation of IRE1α and a slight upregulation of a unique subset of downstream components of ER stress signaling like ERO1α and WFS1. Unexpectedly, this process occurs independently of XBP-1 splicing. Additionally, the mechanism of

IRE1α activation by transient high glucose appears to differ from that of activation by chemical ER stress inducers in regard to BiP dissociation. IRE1 mediated cleavage of XBP-1 mRNA depends on its kinase activity—only wild-type kinase active IRE1 can cleave its mRNA substrate. Upon ER stress, IRE1 dimerizes, which then facilitates *trans*-autophosphorylation and subsequent kinase activation. The coupling of phosphorylation to endoribonucleolytic cleavage, however, has remained unknown.

In 2003, researchers found a hint to this mechanism when they attempted to inhibit IRE1's kinase activity via a mutation in the ATP binding pocket to allow the binding of a bulky kinase inhibitor[120]. Instead of inhibiting IRE1's endoribonuclease activity, however, the inhibitor actually stimulated it. This finding, along with the recent elucidation of the crystal structure of IRE1 [46, 121], reveals that the activation of the endoribonuclease domain is triggered by a conformational change in the kinase domain, promoted by *trans*-autophosphorylation of a kinase activation loop.

These and other studies have also indicated that it is possible that BiP binding to IRE1 negatively regulates XBP-1 splicing. IRE1 endoribonuclease activity is attenuated in mutants where BiP dissociation is impaired [122]. Also, mutations in IRE1 that abolish the BiP binding site render IRE1 hypersensitive to stress, [45] further supporting the idea that BiP binding to IRE1 may serve to dampen activation of downstream targets of IRE1 (i.e.XBP-1). Lastly, IRE1 mutants unable to dissociate from BiP can still sense stress caused by unfolded proteins [46]. These previous findings support our observation that BiP is still bound to IRE1 under transient high glucose treatment, and under these conditions we see no XBP-1 splicing. BiP association with IRE1 may inhibit the

conformational change required to activate the nuclease domain, yet still activate an XBP-1 independent signaling pathway. The mild ER stress caused by glucose-mediated stimulation of proinsulin biosynthesis may not be severe enough to cause BiP dissociation and subsequent XBP-1 splicing, although IRE1's interaction with the unfolded proinsulin may still induce dimerization and *trans*-autophosphorylation.

Although BiP binding and release appears to not be a requirement for control of IRE1 activity, ours and others' work indicates it likely provides a regulatory role under extreme activation conditions, when the pool of free BiP in the ER lumen becomes severely depleted. These conditions may include non-physiological experimental conditions using chemical ER stress inducers like tunicamycin, or upon prolonged UPR induction (i.e. as in Chapter III with chronic high glucose induced ER stress). BiP release under such conditions could serve to induce a conformational change that allows activation of IRE1's nuclease domain. Conversely, BiP binding may dampen activation of IRE1 under conditions of mild unfolded protein accumulation (i.e., transient high glucose-mediated stimulation of proinsulin biosynthesis) through inhibition of the conformational change required for nuclease domain activation.

IRE1α phosphorylation and mild upregulation of a subset of ER stress response genes by transient high glucose, without the accompanying XBP-1 splicing suggests several possibilities. IRE1α phosphorylation and mild upregulation of a subset of ER stress response genes by transient high glucose may, in fact, cause a very small amount of XBP-1 splicing, undetectable by our methods, but sufficient enough to cause the very mild upregulation of certain ER response genes. Alternatively, under mild ER stress

conditions, the unspliced XBP-1 mRNA may be preferentially translated, and may be capable of weakly activating ER stress response genes promoters. Another idea is that IRE1α may have an additional role in β-cells. One emerging possibility comes from recent evidence indicating that protein folding, ER stress, and production of reactive oxygen species (ROS) are closely coupled to one another [92]. Proper protein folding and disulfide bond formation depends on the redox status inside the ER lumen. Mispairing of cysteine residues and inappropriate disulfide bond formation prevents proteins like insulin (which requires 3 disulfide bonds) from attaining their native conformation. Approximately 25% of the ROS generated in a cell may result from formation of disulfide bond formation in the ER, and thus proteins that have multiple disulfide bonds are expected to be more prone to generating ROS [123]. Because β-cells must fold a lot of insulin, they may also be especially sensitive to perturbations of the oxidizing ER environment. Activity of the oxidoreductase ERO1α is tightly coupled with the protein-folding load in the ER [92]. Thus, it is possible that IRE1α may play an important role in the maintenance of the oxidizing environment of the ER, perhaps through upregulation of a variety of different redox enzymes, like ERO1, and other small molecule oxidants. Alterations in redox status and generation of ROS through proinsulin disulfide bond formation could directly affect β-cell ER homeostasis and proinsulin folding. Elucidating the relationship between ER stress and oxidative protein folding is indeed a significant area for future research.

In both Chapter II and III we show strong evidence that IRE1α phosphorylation is induced by exposure to high glucose. Our phospho-IRE1α antibody recognizes a specific

motif, (VGRH(pS)FSRRSG) which is conserved from yeast to humans. In pancreatic β-cells, this site is phosphorylated in response to treatment with many different types of ER stress inducers, including tunicamycin, thapsigargin, and high glucose (which requires glucose metabolism). However, it is likely that other phosphorylation sites exist in the kinase activation loop of IRE1α. It is possible that combinations of phosphorylation of these sites in response to different stimuli may have different biological functions, or may even influence the dimerization/oligomerization state of IRE1. Indeed, there is some disagreement in the field as to whether IRE1 can form higher order oligomers, or whether it strictly exists as a dimer in its active state. Emerging evidence points to yes, that dimers of IRE1 may actually be tethered by unfolded proteins forming active oligomers, and that the biological functions and signaling pathways in these various states may differ [46, 124]. This is an intriguing possibility that requires further investigation.

Our study demonstrates both a physiological and pathological role for Ire1α signaling in pancreatic β-cells (Figure 4.1). IRE1 plays an essential role in the positive regulation of insulin biosynthesis, which indicates that physiological ER stress has a beneficial effect in pancreatic β-cells. We therefore add β-cells to a growing list of secretory cells (plasma cells [51, 52], T-cells [125], trophoblast cells [126]) that utilize ER stress signaling pathways for beneficial processes, differentiation, and normal day-to-day function. We also identify glucose as a physiological activator of ER stress signaling and recommend its use for future studies of the UPR and perhaps unknown signaling cascades not revealed by the previous use of severe stress inducing chemicals.

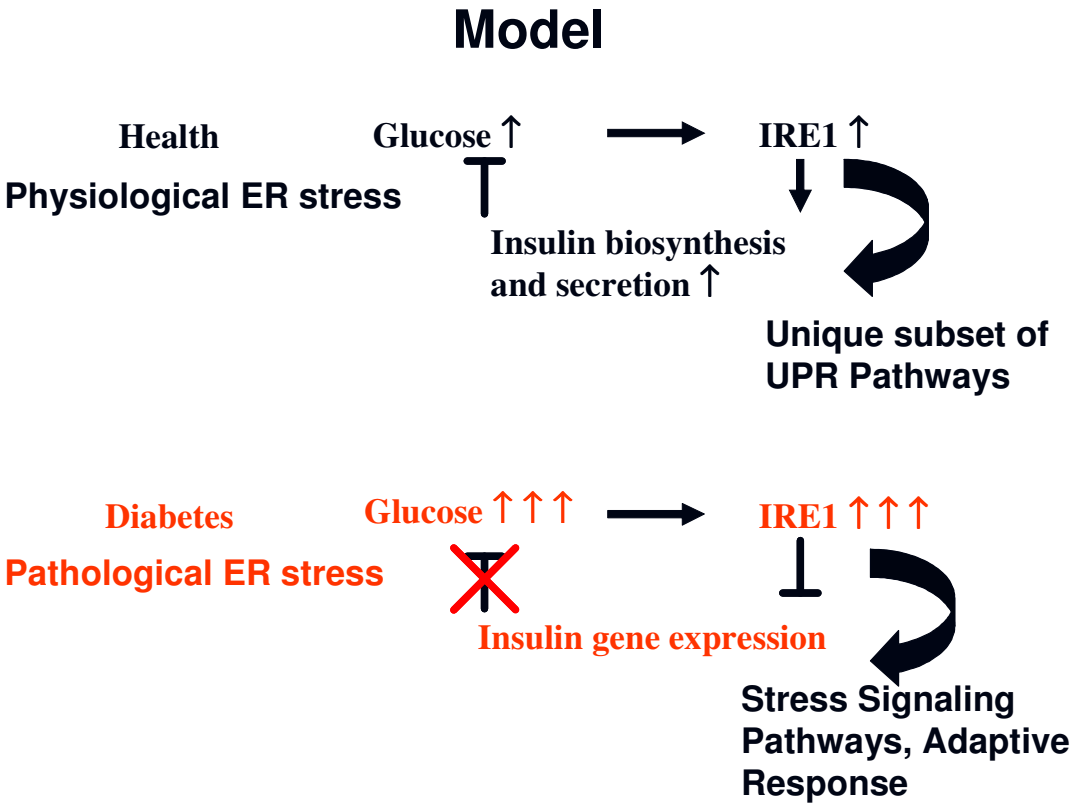

Figure 4.1. Model of physiological and pathological IRE1 activation in pancreatic β-cells
In a healthy individual, consuming food causes an acute increase in blood glucose concentration. This results in insulin secretion from the β-cells in the pancreas and stimulates proinsulin biosynthesis in the ER. Here we present a model whereby glucose-mediated stimulation of insulin biosynthesis promotes physiological ER stress because it imposes a load of insulin on the protein folding and processing machinery of the ER. This has a beneficial effect in β-cells because it results in activation of IRE1 and a slight upregulation of a unique subset of downstream components of ER stress signaling like WFS1, ERO1 and PDI, which aid in the oxidation and folding of the insulin molecule.

In a diabetic individual, hyperglycemia usually occurs due to resistance to the actions of insulin. In this model, chronic hyperglycemia puts severe stress on the cell in a futile attempt to enhance insulin biosynthesis and secretion. The ER of the β-cell may become unable to process and fold the large amounts of insulin mRNA targeted to the ER. Under these pathological ER stress conditions, the ribonuclease activity of IRE1 may now selectively target insulin mRNA for degradation to reduce the workload on the stressed ER of the exhausted β-cell. Our model suggests that this may be an adaptive response designed to preserve the homeostasis of the β-cell. Unfortunately this preservation occurs at the expense of insulin production and secretion.

However, we also show that prolonged activation of IRE1 signaling negatively affects β-cells, in that it results in a decrease in insulin gene expression. Our work implicates chronic ER stress in the mechanisms of glucose toxicity, however, we provide evidence that the IRE1α mediated decrease in insulin mRNA may be an adaptive response β-cells have acquired to protect themselves from ER stress induced death. We also believe our findings further contribute to the understanding of the complex mechanisms of β-cell dysfunction in the progression to Type 2 diabetes (T2D).

The normal pancreatic β-cell's response to a chronic caloric excess and obesity-associated insulin resistance is hypersecretion of insulin in order to maintain normal blood sugar levels (β-cell compensation). It seems likely that IRE1α is active in a physiological role under these conditions, helping to maintain favorable conditions in the ER that allows for the increase in insulin biosynthesis required to keep up with the hypersecretion. Studies of people that develop T2D show a rise in insulin levels in the normoglycemic and prediabetes phases that keep blood sugar levels near normal, despite the insulin resistance (β-cell compensation). T2D only develops in subjects that are unable to sustain the β-cell compensatory response (β-cell failure) and this is accompanied by a decline in insulin levels.

Compensation fails in subjects that have "susceptible", as opposed to "robust", islets. Compensation involves expansion of β-cell mass, enhanced insulin biosynthesis, and increased responsiveness of nutrient-secretion coupling [127]. Insulin biosynthesis is known to be regulated by acute changes in nutrient availability [87], but very little is known about longer-term adaptations in transcriptional/translational regulation of insulin

production to situations such as chronic overnutrition. Our work suggests ER stress signaling via IRE1α enhanced insulin biosynthesis may be important in the process of β-cell compensation.

β-cell failure occurs in islets that are undergoing the processes of compensation for insulin resistance. One model of β-cell failure suggests either one, or a small number, of β-cell defects act as the weak link in the processes of β-cell compensation that initiate β-cell dysfunction [77]. The likely mechanisms of early β-cell demise are numerous, including mitochondrial dysfunction, oxidative stress, ER stress, dysfunctional triglyceride/FFA (TG/FFA) cycling, and glucolipotoxicity. β-cell compensation may progress to β-cell failure in subjects who are genetically susceptible to ER stress or have a defect in ER stress signaling. One intriguing possibility our work suggests is that chronic activation of the IRE1α signaling pathway initiates a β-cell protective response. During the switch from compensation to failure, the insulin resistance worsens, hyperglycemia occurs, and the ER of the β-cells can no longer keep up with the large amounts of insulin mRNA targeted to the ER to enhance insulin biosynthesis and secretion. In this scenario, the ribonuclease activity of IRE1α may now selectively target insulin mRNA for degradation to reduce the workload on the stressed ER of the exhausted β-cell. This may preserve the homeostasis of the β-cell, but at the expense of insulin production and secretion.

For cells to survive under chronic ER stress conditions like prolonged hyperglycemia or hyperproduction and secretion of insulin, they must have a mechanism by which ER stress can be continuously tolerated. A small number of cells may die, but

the majority of cells must survive and adapt to the stressful stimulus [20]. In one human study, T2D subjects had a 41% loss of β-cell volume compared with weight-matched controls. Furthermore, the decreased volume was not a consequence of reduced β-cell proliferation but was associated with increased β-cell death by apoptosis [4]. This adaptation may be crucial for the preservation of β-cells and may, in part, explain why the remaining 60% of β-cells of Type 2 diabetic patients with chronic hyperglycemia stop producing insulin without simply undergoing apoptosis.

What about the β-cells that do undergo apoptosis? It has been suggested that two signaling components of ER stress, IRE1-JNK, and CHOP, have important roles in β-cell death mediated by ER stress. It has been shown that in the presence of chronic ER stress, IRE1 activates JNK through ASK1 and elicits apoptosis [53, 72]. This pathway may block the functions of the anti-apoptotic BCL2 family members Bcl2 and Bcl-XL by phosphorylating them, thus causing apoptosis in β-cells. Also, the IRE1-JNK pathway has been shown to enhance the functions of two pro-apoptotic factors, Bax and Bak, and can lead to apoptosis in some cell types [73]. Although some studies have indicated that JNK activation contributes to cytokine-induced β-cell death [128], the function of ER stress–mediated JNK activation in the death of β-cells has not been studied extensively and must be defined. Nevertheless, our findings in Chapter II indicate that the IRE1-JNK pathway is activated by chronic high glucose treatment. Susceptible β-cells that cannot reduce their ER workload via insulin mRNA degradation may undergo apoptosis via the IRE1-JNK pathway. Our work also suggests that a transient increase in insulin biosynthesis activates IRE1 signaling in β-cells and mildly upregulates the pro-apoptotic

factor CHOP. Thus, a prolonged increase in insulin biosynthesis, such as what occurs in patients with insulin resistance, may strongly upregulate CHOP and elicit apoptosis in this manner.

Much of our work has focused on the importance of ER stress in the pathogenesis of Type 2 diabetes and rare forms of Type 1 diabetes such as Wolfram syndrome. However, ER stress-mediated β-cell death may also contribute to the pathogenesis of autoimmune diabetes. Since the baseline level of ER stress is high in pancreatic β-cells, it is possible that a slight additional increase in ER stress by environmental or genetic factors leads to β-cell death in patients who are genetically susceptible to ER stress. β-cells dying as a consequence of a high level of ER stress may contain proteins with abnormal conformations (i.e., misfolded proteins). The engulfment of ER stress-induced apoptotic β-cells by dendritic cells in the islets may stimulate the maturation of β-cell-reactive T-cells in draining lymph nodes and lead to T-cell-mediated autoimmune destruction by "neo-autoantigens" derived from misfolded proteins [129].

Our work, and recent work from other labs, suggests that IRE1α may be an attractive therapeutic target for the treatment of people with diabetes. Recent experiments show a single missense mutation in the ATP-binding pocket of mouse IRE1α's kinase domain sensitizes it to the ATP-competitive inhibitor 1NM-PP1, and subordinates its RNase activity to the drug. Engagement of the drug-sensitized IRE1α kinase in this manner protects cells against ER stress induced cell death [130]. Thus kinase inhibitors of IRE1α may be useful for both preventing degradation of insulin mRNA, and altering the apoptotic outcome to ER stress, and could possibly be developed into drugs to treat ER

stress-related diseases. However, our work indicates that extreme caution must be taken if attempting to regulate IRE1α signaling; although mild activation of IRE1 has a beneficial effect on pancreatic β-cells, strong activation of IRE1 has a harmful effect on pancreatic β-cells, and could reduce insulin production in our body. The ideal drug to enhance insulin biosynthesis, and perhaps, aid or extend the β-cell compensation stage, would cause only mild activation of IRE1. If degradation of insulin mRNA is, in fact, a preservation attempt, then inhibiting IRE1 activity under chronic hyperglycemia conditions may not be useful at all, as it may initiate apoptosis if the ER becomes overwhelmed with insulin processing. Our work indicates that an IRE1 "inhibitor" drug may have the best effect after the insulin resistance has been controlled, to try to coax β-cells into producing insulin again, or in combination with another drug that helps increase peripheral cells' sensitivity to insulin. The "two-toned" nature of IRE1α activation, while extremely promising, suggests there is still much more to understand about this extraordinary enzyme.

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