Regulation of insulin biosynthesis in pancreatic beta cells by an endoplasmic reticulum-resident protein kinase IRE1

Kathryn L. Lipson, Sonya G. Fonseca, Shinsuke Ishigaki, Linh X. Nguyen, Elizabeth Foss, Rita Bortell, Aldo A. Rossini, and Fumihiko Urano

1 Program in Gene Function and Expression
2 Program in Molecular Medicine and
3 Department of Medicine
University of Massachusetts Medical School, Worcester, Massachusetts 01605
*Correspondence: fumihiko.urano@umassmed.edu

Summary

In pancreatic β cells, the endoplasmic reticulum (ER) is an important site for insulin biosynthesis and the folding of newly synthesized proinsulin. Here, we show that IRE1α, an ER-resident protein kinase, 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 hinders insulin biosynthesis. IRE1 activation by high glucose does not accompany XBP-1 splicing and BiP dissociation but upregulates its target genes such as WFS1. Thus, IRE1α signaling activated by transient exposure to high glucose uses a unique subset of downstream components and has a beneficial effect on 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. IRE1 signaling is therefore a potential target for therapeutic regulation of insulin biosynthesis.

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. As preproinsulin, a precursor of insulin, is synthesized in the cytoplasm with a signal peptide, it is cotranslationally translocated into the lumen of the ER through interaction between the signal peptide and the signal recognition particle on the ER membrane. The signal peptide of preproinsulin is cleaved in the ER and proinsulin is produced. In the lumen of the ER, proinsulin undergoes meticulous protein folding whereby three disulfide bonds are formed. Properly folded proinsulin is then delivered to the Golgi apparatus and packaged into secretory granules. The conversion of proinsulin to insulin takes place in the secretory granules. Mature insulin is then released by exocytosis (Rhodes, 2004; Rhodes et al., 2005). 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 folding capacity of the ER negatively affects the homeostasis of β cells and leads to ER stress. (Harding et al., 2000a; Kaufman, 2002).

The unfolded protein response (UPR) is an intracellular system that mitigates ER stress (Harding et al., 2002; Kaufman et al., 2002; Mori, 2000). 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 consequent 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 such as ER degradation-enhancing α-mannosidase-like protein (EDEM) and genes that are important for protein folding such as protein disulfide isomerase-P5 (Calfon et al., 2002; Lee et al., 2003; Yoshida et al., 2003). IRE1-XBP-1 signaling is also important for expansion of the ER (Shaffer et al., 2004; Sriburi et al., 2004), which, in turn, is needed for the differentiation of secretory cells (Iwakoshi et al., 2003a, 2003b; Reimold et al., 2001). 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 (Nishitoh et al., 2002; Urano et al., 2000).

Mammalian cells have two types of IRE1, α and β. IRE1α is expressed ubiquitously, having high levels of expression in the pancreas and placenta (Tirasophon et al., 1998), while IRE1β is expressed only in epithelial cells of the gastrointestinal tract (Bertolotti et al., 2001; Wang et al., 1998). 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 (Calfon et al., 2002; Iwakoshi et al., 2003a, 2003b; Lee et al., 2005; Reimold et al., 2001). This suggests that physiological ER stress is necessary for the differentiation of secretory cells. However, a physiological inducer of ER stress has not been studied extensively. Also, no studies have reported that physiological activation of IRE1 has a function in regulated synthesis of secretory proteins. We undertook this study to test the hypothesis that a high glucose level physiologically activates IRE1 and a unique subset of downstream components of IRE1. Under these conditions, IRE1 activation has
a beneficial effect aiding in the enhancement of postprandial proinsulin biosynthesis in pancreatic β cells. In contrast, chronic exposure of β cells to high glucose causes ER stress and hyperactivation of IRE1, leading to insulin gene suppression. Therefore, IRE1 hyperactivation and ER stress elicited by chronic hyperglycemia have a harmful effect in pancreatic β cells. Our results show that IRE1 signaling is a potential target for therapeutic regulation of insulin biosynthesis.

Results

IRE1α in pancreatic islets is activated by high glucose

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 (Tirasophon et al., 1998) expressed in COS7 cells. The antibody specifically detected wild-type IRE1α, which is autophosphorylated by overexpression (Tirasophon et al., 2000, 1998), but did not recognize the kinase-inactive mutant (Figure 1A, upper panel). The expression level of kinase-inactive K599A IRE1α was confirmed by an antibody that can recognize both phosphorylated and unphosphorylated IRE1α (Uranoto et al., 2000) (Figure 1A, lower panel). The expression level of kinase-inactive IRE1α was much higher than that of wild-type IRE1α as previously reported (Tirasophon et al., 1998; Uranoto et al., 2000).

Using anti-phospho-specific IRE1α antibody, we measured IRE1α activation levels in mouse pancreatic islets, which have a primary function in the metabolism of glucose by secreting 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 hr increased the phosphorylation level of IRE1α compared to 2.5 mM (Figure 1B, left 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 hr and measured IRE1α phosphorylation. Treatment with 10 mM glucose also increased IRE1 phosphorylation (Figure 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, indicating 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 hr 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 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 hr using real-time polymerase chain reaction (PCR). Treatment with 16.7 mM glucose caused an increase in the expression of BiP, Wfs1, Erp1, Chop, and total Xbp-1 (Figure 2A), markers for ER stress. Wfs1 is a target gene of IRE1 signaling in pancreatic β cells (Fonseca et al., 2005). Therefore, we expected that Xbp-1 splicing would also be activated in islets treated with high glucose. Unexpectedly, Xbp-1 splicing was not activated by high glucose treatment (Figure 2A, lowest panel, right side). To confirm this observation, we treated mouse islets with 2.5 mM and 16.7 mM glucose or tunicamycin, a classic inducer of ER stress for 1 hr, 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 (Figures 2B and 2C). However, only tunicamycin treatment, and not high glucose treatment, induced Xbp-1 splicing (Figure 2C). These results suggest that high glucose treatment induces activation of IRE1, that is not accompanied by Xbp-1 splicing.

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 β cells, which produce insulin. Thus, the activation of IRE1α is beneficial for improving the function of pancreatic islets.
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, we found that the IRE1α phosphorylation level is linked to the insulin secretion level in insulinoma cells, INS-1. Treatment of INS-1 cells with high glucose (10, 20, and 25 mM) for 3 hr induced both insulin secretion and IRE1α phosphorylation (Figure 3A), suggesting that IRE1α is a positive regulator of insulin secretion and biosynthesis. 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. 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 treatment with 2DG decreased IRE1α phosphorylation (Figure 3B), 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 decreased IRE1α phosphorylation (Figure 3B). 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 ER stress**

To test the extent to which IRE1α is activated by different stimuli, we treated INS-1 cells with a series of insulin secretagogues, including high glucose, arginine, tolbutamide, KCl, and GLP-1.
BiP (Bertolotti et al., 2000). Under normal, unstressed conditions, IRE1 activation is controlled by an ER-resident molecular chaperone, but do not strongly activate known insulin secretagogues. We hypothesized that IRE1 positively regulates insulin secretion at the biosynthesis level. To study the role of IRE1 in proinsulin biosynthesis directly, we inhibited the expression of IRE1 in INS-1 cells, using siRNA specific for IRE1. This suppression of IRE1 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 5A). We also examined the dominant negative effect of mutant IRE1, which lacks kinase activity (Tirasophon et al., 1998; Urano et al., 2000). The expression of mutant IRE1, in which the ATP binding domain site at the lysine residue is replaced with alanine, decreased both the phosphorylation level of endogenous IRE1 and the insulin content in INS-1 cells (Figure 5B). To determine whether IRE1 controls insulin biosynthesis at the translation and protein-folding level, we measured proinsulin biosynthesis in INS-1 cells transfected with siRNA for IRE1 by pulse-chase. IRE1 suppression was confirmed by immunoblot with anti-IRE1 antibody (Figure 5C, second panel). This suppression of IRE1 dramatically decreased proinsulin biosynthesis, indicating that IRE1 functions in insulin biosynthesis at the translation and protein folding level (Figure 5C, top panel). However, IRE1 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 IRE1 by siRNA does not suppress general protein translation, we measured total protein biosynthesis by pulse-chase, finding that total protein biosynthesis was not significantly affected by IRE1 siRNA (Figure 5C, bottom panel). These results indicate that IRE1 signaling has a beneficial effect on pancreatic β cells, enhancing insulin biosynthesis. To determine the effect of IRE1 on glucose-stimulated insulin secretion (GSIS: the ratio between secreted insulin and cellular insulin content), we measured GSIS in IRE1-siRNA-transfected β cells. We transfected INS-1 832/13 cells with siRNA for IRE1 and preincubated the cells with 5 mM glucose overnight, then, stimulated the cells with 5 mM or 10 mM glucose for 1 hr and measured GSIS by radioimmunoassay (RIA). GSIS was not suppressed in IRE1-siRNA-β cells as compared to control cells with 5 mM glucose (Figure 5D, upper panel). GSIS was slightly decreased in IRE1-siRNA-β cells with 10 mM glucose. These results suggest that IRE1 has an important function in proinsulin biosynthesis, but not in insulin secretion. Additionally, siRNA knockdown of IRE1 signaling decreases insulin biosynthesis

Because IRE1 signaling is important for protein folding in the ER, we hypothesized that IRE1 positively regulates insulin secretion at the biosynthesis level. To study the role of IRE1 in proinsulin biosynthesis directly, we inhibited the expression of IRE1 in INS-1 cells, using siRNA specific for IRE1. This suppression of IRE1 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 5A). We also examined the dominant negative effect of mutant IRE1, which lacks kinase activity (Tirasophon et al., 1998; Urano et al., 2000). The expression of mutant IRE1, in which the ATP binding domain site at the lysine residue is replaced with alanine, decreased both the phosphorylation level of endogenous IRE1 and the insulin content in INS-1 cells (Figure 5B). To determine whether IRE1 controls insulin biosynthesis at the translation and protein-folding level, we measured proinsulin biosynthesis in INS-1 cells transfected with siRNA for IRE1 by pulse-chase. IRE1 suppression was confirmed by immunoblot with anti-IRE1 antibody (Figure 5C, second panel). This suppression of IRE1 dramatically decreased proinsulin biosynthesis, indicating that IRE1 functions in insulin biosynthesis at the translation and protein folding level (Figure 5C, top panel). However, IRE1 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 IRE1 by siRNA does not suppress general protein translation, we measured total protein biosynthesis by pulse-chase, finding that total protein biosynthesis was not significantly affected by IRE1 siRNA (Figure 5C, bottom panel). These results indicate that IRE1 signaling has a beneficial effect on pancreatic β cells, enhancing insulin biosynthesis. To determine the effect of IRE1 on glucose-stimulated insulin secretion (GSIS: the ratio between secreted insulin and cellular insulin content), we measured GSIS in IRE1-siRNA-β cells. We transfected INS-1 832/13 cells with siRNA for IRE1 and preincubated the cells with 5 mM glucose overnight, then, stimulated the cells with 5 mM or 10 mM glucose for 1 hr and measured GSIS by radioimmunoassay (RIA). GSIS was not suppressed in IRE1-siRNA-β cells as compared to control cells with 5 mM glucose (Figure 5D, upper panel). GSIS was slightly decreased in IRE1-siRNA-β cells with 10 mM glucose. These results suggest that IRE1 has an important function in proinsulin biosynthesis, but not in insulin secretion. Additionally, siRNA...
knockdown of IRE1α had no effect on insulin gene expression as measured by real-time PCR (results not shown).

Sustained IRE1 signaling decreases insulin gene expression

Treatment of β cells with high physiological concentrations of glucose for 1 hr 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 hr 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) caused XBP-1 splicing, indicating that chronic hyperglycemia induces typical IRE1 activation and ER stress (Figure 6A). We 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. 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 6B, lanes 2 and 5). Treatment with 25 mM glucose further increased the phosphorylation level of IRE1α, but decreased the cellular insulin content (Figure 6B, lanes 3 and 6), suggesting that hyperactivation of IRE1α may decrease insulin biosynthesis. 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 6C), suggesting that sustained activation of IRE1α (i.e., ER stress) may decrease insulin biosynthesis at the transcription level. However, these observations may 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 roles in development of antibody-secreting plasma cells, hepatocytes, and exocrine pancreatic cells (Lee et al., 2005; Reimold et al., 2000, 2001). Here, we have shown that regulated activation of IRE1α under physiological settings has an important function in pancreatic β cells. Postprandial hyperglycemia activates IRE1α, leading to the enhancement of proinsulin biosynthesis. We have therefore determined that high 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.”
The components of SCAEF probably have important functions in proinsulin biosynthesis in pancreatic β cells. For example, an ER-resident oxidoreductase, ERO1α, is upregulated by transient high glucose and functions in proinsulin folding (Lipson and Urano, unpublished observations). Since ERO1α is an activator for PDI, which has a crucial role in disulfide bond formation (Tu and Weissman, 2004), 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 (Harding et al., 2001). PERK is highly expressed in pancreatic islets (Harding et al., 1999; Shi et al., 1998). 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 (Harding et al., 2000b). 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 (Harding et al., 2001). 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.

\[\text{Figure 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. Shown is a typical result of an experiment repeated three times.
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 hr, then with 0 mM glucose for 1 hr. The cells were stimulated with KRB buffer containing 25 mM glucose for 3 hr. Expression levels of total Ire1α, phosphorylated Ire1α (P-Ire1α), insulin, and actin expression were measured by immunoblot. Shown is a typical result of an experiment repeated three times.
C) INS-1 cells were transfected with siRNA specific for rat ire1α (final concentration, 20 nM) or siRNA-scramble (Qiagen) as a control. After transfection, the cells were pretreated with 11 mM glucose for 18 hr, then stimulated with 16.7 mM glucose and incubated with [35S]methionine for 1 hr. [35S]-labeled proinsulin was immunoprecipitated and subjected to SDS-PAGE analysis followed by visualization with a phosphorimager. [35S]-labeled total protein was also analyzed by SDS-PAGE followed by visualization with a phosphorimager. 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 hr, then stimulated with 5 mM glucose or 10 mM glucose for 1 hr. 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.
However, it has been reported that deregulated eIF2α phosphorylation also leads to declining glucose homeostasis (Scheuner et al., 2001). Therefore, the derepression of proinsulin biosynthesis in Perk knockout mice may reflect the defect in glucose homeostasis. Mutations in the EIF2AK3 gene encoding PERK have been reported in Wolcott-Rallison syndrome, a rare form of juvenile diabetes (Delepine et al., 2000). PERK knockout mice also develop diabetes as a consequence of a high level of ER stress in the pancreas (Harding et al., 2001). 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.

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 (Bertolotti et al., 2000). 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 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 (Kimata et al., 2004). Also, Walter and his colleagues 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. 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 (Credle et al., 2005). 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 are proapoptotic components of the UPR. However, we were surprised to find that XBP-1 also is not spliced by high-glucose activation 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 recent 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 (Lee et al., 2005). It is possible that in endocrine pancreatic tissue, IRE1 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 by 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 due to insulin resistance. Glucose toxicity is defined as nonphysiological and potentially irreversible β cell damage caused by chronic exposure to supraphysiological glucose concentrations (Leahy, 2004; Robertson et al., 2004). 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 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 JNK signaling (Ozcan et al., 2004), 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 (Nishitoh et al., 2002). These findings indicate that activation of IRE1 by pathological conditions, such as obesity, polyglutamine accumulation, and chronic hyperglycemia, is harmful to cells.

In contrast, 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.

**Experimental procedures**

**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 (Amaza Biosystems, Gaithersburg, MD) was used to transfect small interfering RNA (siRNA) for IRE1a into INS1 cells. At QIAGEN (Valencia, CA), siRNAs for rat IRE1a were designed and synthesized: for rat IRE1a, AAGGCAGATGATCAGACTTT.

**ELISA**

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

**Glucose-stimulated insulin secretion**

INS-1 832/13 cells were transfected with siRNA for IRE1a (final concentration: 20 nM) and preincubated with 5 mM glucose overnight, then stimulated with 5 mM or 10 mM glucose for 1 hr. We measured the insulin secretion level and cellular insulin content by radioimmunoassay (RIA) (Linco Diagnostics, St. Charles, MO). The ratio between secreted insulin and cellular insulin content was calculated.

**Immunoblottting and immunoprecipitation**

Cells were lysed in ice-cold M-PER buffer (PIERCE, Rockford, IL) containing protease inhibitors for 15 min on ice then the lysates were cleared by centrifuging the cells at 13,000 × 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 IRE1a antibody was generated from bulk antisera 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 (pS) FERRRS. This phosphopeptide was synthesized, multi-link-conjugated to KLH, and used to immunize 2SF rabbits. Rabbit anti-total IRE1a antibody (B9134) was generated using a peptide, EGWIAPLSEMDEDCK. 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-elf2α antibody was purchased from Sanat Cruz Biotechnology (Santa Cruz, CA), anti-phospho-elf2α 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).

**Insulin biosynthesis**

INS-1 cells were pretreated with 5 mM glucose in RPMI for 18 hr, then with 2.5 mM glucose in KR buffer (135 mM NaCl, 3.6 mM KCl, 10 mM Hepes [pH 7.4]), 5 mM NaHCO₃, 0.5 mM NaH₂PO₄, 0.5 mM MgCl₂, 1.5 mM CaCl₂) for 1 hr. The cells were stimulated with KR buffer containing 16.7 mM of glucose for 1 hr. 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**

Proinsulin biosynthesis was analyzed by proinsulin immunoprecipitation of [³⁵S] methionine-radioabeled INS-1 lysates as described (Alarcon et al., 2002; Wicksteed et al., 2003, 2001). Immunoprecipitated [³⁵S]-labeled proinsulin was then subjected to SDS-PAGE and visualized by phosphomager.

**Isolating islets from mouse pancreata**

Mice were anesthetized by intraperitoneal injection of sodium pentobarbital. Pancreatic islets are then isolated by pancreatic duct injection of 500 U/ml of collagenase solution followed by digestion at 37 °C for 40 min 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-phenol extraction method and reverse transcribed using 1 µg 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 min, 95 °C for 10 min, then 40 cycles at 95 °C for 15 s, and at 60 °C for 1 min.
Acknowledgments

We thank Dr. Christopher Rhodes, Dr. Gordon Weir, Dr. Susan-Bonner Weir, Dr. Michael Czeck, Dr. Silvia Corvera, Dr. Michael Green, and Jeanne Cole for comments on the manuscript, Karen Sargent, Linda Leehy, and Elaine Norowski for technical assistance, Dr. Christopher Newgard for INS-1 and INS-1 832/13 cells, Dr. David Ron for anti-total IRE1 antibody, and Dr. Randall Kaufman for human IRE1α cDNA. Research in the laboratory of F.U. is supported by an NIH R01DK067493 grant, NIH-NIDDK Diabetes and Endocrinology Research Center at the UMass Medical School, an American Diabetes Association Innovation Grant, a Juvenile Diabetes Research Foundation Innovation Grant, a Worcester Foundation Award, and an Iacocca Foundation Grant.

Received: February 23, 2006
Revised: June 6, 2006
Accepted: July 21, 2006
Published: September 5, 2006

References


