

UNIVERSITA' DEGLI STUDI DI PARMA

Dottorato di ricerca in Fisiopatologia sistemica

Ciclo XXVIII

**Role of heme regulated eIF2 α kinase in
pancreatic beta cell function and viability**

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Whatever you can do or dream you can, begin it.

Boldness has genius, power and magic in it.

Goethe

Abstract

The eukaryotic translation initiation factor 2 alpha (eIF2 α) is part of the initiation complex that drives the initiator amino acid methionine to the ribosome, a crucial step in protein translation. In stress conditions such as virus infection, endoplasmic reticulum (ER) stress, amino acid or heme deficiency eIF2 α can be phosphorylated and thereby inhibit global protein synthesis. This adaptive mechanism prevents protein accumulation and consequent cytotoxic effects. Heme-regulated eIF2 α kinase (HRI) is a member of the eIF2 α kinase family that regulates protein translation in heme deficiency conditions. Although present in all tissues, HRI is predominantly expressed in erythroid cells where it remains inactive in the presence of normal heme concentrations. In response to heme deficiency, HRI is activated and phosphorylates eIF2 α decreasing globin synthesis. This mechanism is important to prevent accumulation of heme-free globin chains which cause ER stress and apoptosis. RNA sequencing data from our group showed that in human islets and in primary rat beta cells HRI is the most expressed eIF2 α kinase compared to the other family members. Despite its high expression levels, little is known about HRI function in beta cells. The aim of this project is to identify the role of HRI in pancreatic beta cells. This was investigated taking a loss-of-function approach.

HRI knock down (KD) by RNA interference induced beta cell apoptosis in basal condition. HRI KD potentiated the apoptotic effects of palmitate or proinflammatory cytokines, two in vitro models for type 2 and type 1 diabetes, respectively. Increased cytokine-induced apoptosis was also observed in HRI-deficient primary rat beta cells. Unexpectedly, we observed a mild increase in eIF2 α phosphorylation in HRI-deficient cells. The levels of mRNA or protein expression of C/EBP homologous protein (CHOP) and activating transcription factor 4 (ATF4) were not modified. HRI KD cells have decreased spliced X-box binding protein 1 (XBP1s), an important branch of the ER stress response.

However, overexpression of XBP1s by adenovirus in HRI KD cells did not protect from HRI siRNA-induced apoptosis. HRI deficiency decreased phosphorylation of Akt and its downstream targets glycogen synthase kinase 3 (GSK3), forkhead box protein O1 (FOXO1) and Bcl-2-associated death promoter (BAD). Overexpression of a constitutively active form of Akt by adenovirus in HRI-deficient beta cells partially decreased HRI KD-mediated apoptosis. Interestingly, BAD silencing protected from apoptosis caused by HRI deficiency. HRI silencing in beta cells also induced JNK activation.

These results suggest an important role of HRI in beta cell survival through modulation of the Akt/BAD pathway. Thus, HRI may be an interesting target to modulate beta cell fate in diabetic conditions.

Acknowledgements

I would like to thank my promoter Prof Alessandra Dei Cas for supporting me and my work during these past three years. It has been an honor to be her first PhD student. I also wish to express my sincere gratitude to my promoter Prof Miriam Cnop for providing me the opportunity to join her research group at the Center of Diabetes Research at the Université libre de Bruxelles for her helpfulness, knowledge and precious scientific advice.

My profound thanks also go to Dr Daniel A. Cunha whose expertise, knowledge, understanding, enthusiasm and patience encouraged and helped me throughout the course of this project. He is the best scientific mentor I could have wished for my PhD experience.

I would like to express my deepest appreciation to Prof Riccardo Bonadonna, who offered his continuous advice and encouragement. His love for research and his support were driving forces for my PhD project.

I also thank Prof Enrico Maria Silini, coordinator of Systemic Physiopathology PhD School.

Furthermore, I thank SID – Società italiana di diabetologia – for its financial support to this project.

Special thanks go to my friend and colleague Dr Valentina Spigoni who gave me continuous support and help from the very first day of my PhD. Her passion and intense dedication to the scientific research encouraged me to take up this challenge.

I am thankful to all my friends and colleagues who supported me every single day of these past three years.

Last but not the least, my sincere and profound gratitude goes to my family for supporting and encouraging me to achieve my goals throughout my life.

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Abbreviations

A1	Bcl-2-related protein A1
Akt	Protein Kinase B
APAF-1	Apoptotic Protease Activating Factor-1
ATF	Activating Transcription Factor
ATP	Adenosine triphosphate
BAD	Bcl-2-Associated Death Promoter
Bak	Bcl-2-Antagonist/Killer 1
Bax	Bcl-2-Associated X Protein
Bcl-2	B-Cell Lymphoma 2
Bcl-XL	Bcl-2-extra large
Bcl-W	Bcl-2-like protein 2
Bik	Bcl-2-interacting killer
Bim	Bcl-2-Like Protein 11
BiP	Immunoglobulin heavy-chain binding protein
Bok	Bcl-2-Related Ovarian Killer
BSA	Bovine serum albumin
CAT	Catalase
CHOP	C/Ebp Homologous Protein
DFO	Deferoxamine

DP5	Death Protein 5
dsRBD	Double-stranded RNA Binding Domain
dsRNA	Double-stranded RNA
eIF	Eukaryotic Translation Initiation Factor
ER	Endoplasmic Reticulum
ERAD	Endoplasmic-Reticulum-Associated protein Degradation
ETC	Electron Transport Chain
FACSAria	Autofluorescence-Activated Cell Sorting
FBS	Fetal Bovine Serum
FFAs	Free Fatty Acids
FOXO1	Forkhead Box Protein O1
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GCN2	General Control Non-Derepressible-2
GDP	Guanosine diphosphate
GPx	Glutathione Peroxidase
GSK3	Glycogen Synthase Kinase 3
GTP	Guanosine triphosphate
HisRS	Histidyl-tRNA Synthetase
HO	DNA dyes Hoechst
HRI	Heme-Regulated Inhibitor
Hsp	Heat shock protein

IFN	Interferon
IL	Interleukin
INS	Rat insulin-producing cell line
IRE-1	Inositol-Requiring Kinase-1
JNK	c-Jun N-terminal Kinase
K562	Human myelogenous leukemia line
KD	Knock Down
KO	Knock Out
KRBH	Krebs-Ringer bicarbonate HEPES
LUC	Luciferase
MAPK	Mitogen-Activated Protein Kinase
MIN6	Transgenic C57BL/6 mouse insulinoma cell line
MOI	Multiplicity of infection
MOMP	Mitochondrial Outer Membrane Permeabilization
NF-kB	Nuclear factor kB
Noxa	Phorbol-12-myristate-13-acetate-induced protein 1
NO	Nitric oxide
O2-	Superoxide anion
PCR	Polymerase chain reaction
PERK	(PKR)-like ER Kinase
PI	Propidium iodide

PI3K	Phosphoinositide 3-Kinase
PKR	Protein Kinase double-stranded RNA-Dependent
PUMA	p53 Upregulated Modulator of Apoptosis
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
SOD	Superoxide Dismutase
SP	Site Protease
T1D	Type 1 Diabetes
T2D	Type 2 Diabetes
tBID	truncated BH3 Interacting-Domain Death agonist
Tg	Thapsigargin
TNFR	Tumor Necrosis Factor Receptor
TNF	Tumor Necrosis Factor
TRAF2	(TNFR)-Associated Factor 2
tRNA	transfer RNA
uORF	Upstream-Open Reading Frames
UPR	Unfolded Protein Response
WRS	Wolcott-Rallison Syndrome
XBP1t	X-Box Binding Protein-1 total
XBP1s	X-box Binding Protein 1 spliced

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1. Introduction

1.1 Diabetes mellitus

Diabetes mellitus is a metabolic disorder induced by defective insulin production and action that lead to hyperglycemia¹. In 2015, 415 million people worldwide have diabetes and this estimate is expected to increase to 642 million by 2040². There are two major forms of diabetes; type 1 (T1D) is predominant in younger people and represents 10-15% of worldwide cases. Type 2 (T2D), with 85% of incidence, is predominantly developed in adults in develop countries. Several environmental factors combined to an adverse genetic background, trigger the development of both diabetes forms. T1D is an autoimmune disease possibly triggered by viral infection or endogenous ligands of primitive pattern recognition receptors³. T1D is characterized by mononuclear cell infiltration in the pancreatic islets and consequent chronic inflammatory reaction termed “insulinitis”. T-cells and activated macrophages direct interact with beta cells and secrete proinflammatory cytokines such as interleukin (IL)-1 β , tumor necrosis factor (TNF)- α and interferon (IFN)- γ 1 as well as nitric oxide and oxygen free radicals leading to loss of beta cell mass⁴. In T2D Western diets and sedentary life style are major contributors for its increased incidence^{5,6}. Hyperglycemia develops in insulin resistant individuals who cannot mount a compensatory insulin response⁷. Dietary even-chain saturated free fatty acids, of which palmitate is the most common in man, have been associated

with T2D risk in prospective studies⁸ and cause loss of functional beta cell mass⁹, which is at least in part due to beta cell apoptosis¹⁰. Accumulating evidence suggests that both endoplasmic reticulum (ER) and oxidative stress are important mediators of beta cell dysfunction in T1D and T2D^{11,12}.

The ER is a cell organelle important for biosynthesis, folding and processing of secretory and membrane proteins. Beta cells require an efficient ER since it synthesizes and secretes large quantities of insulin in response to glucose. Stress conditions can disrupt ER homeostasis by loss of chaperones, calcium depletion or accumulation of mutant misfolded proteins in the ER lumen¹³. To cope with the loss of ER homeostasis, cells activate an adaptive signaling network called the unfolded protein response (UPR). The UPR modulates ER stress through different mechanisms: by increased ER chaperone expression to improve folding capacity, decreased synthesis of new proteins to avoid accumulation in ER lumen and increased misfolded protein ejection and degradation. If the UPR fails to reestablish ER homeostasis, apoptosis is triggered¹⁴.

Three main transmembrane ER proteins are activated in the UPR: activating transcription factor (ATF)-6, inositol-requiring kinase-1 (IRE-1) and double-stranded RNA-activated kinase (PKR)-like ER kinase (PERK). The ER chaperone immunoglobulin heavy-chain binding protein (BiP) binds these ER stress transducers to maintain them in a inactive form¹⁵. In the presence of protein accumulation in the ER lumen, BiP is released to assist in correct protein folding, thus inducing activation of the ER stress response. ATF6 translocates to the Golgi

where it is cleaved by site 1 protease (SP1) and SP2 releasing the cytosolic DNA-binding portion. In the nucleus, ATF6 activates chaperone gene transcription, such as BiP¹⁶, and augments the expression of X-box binding protein-1 (XBP1) mRNA, a substrate of IRE-1^{13,17}. Activated IRE-1 catalyzes the alternative splicing of the XBP1 mRNA, leading to translation of its active form that translocates to the nucleus and induces expression of chaperones and endoplasmic reticulum-associated degradation (ERAD) proteins, ultimately improving cell survival¹⁵. In case of severe ER stress, IRE-1 recruits the tumor necrosis factor receptor (TNFR)-associated factor 2 (TRAF2). TRAF2-IRE1 complex activates c-Jun N-terminal kinase (JNK) and caspase-12 leading to apoptosis^{13,14}.

PERK is another important branch of the ER stress response that modulates protein translation by phosphorylation of eukaryotic translation initiation factor (eIF) 2 α . eIF2 α is part of the initiation complex that drives the initiator amino acid methionine to the ribosome, a crucial step in protein translation. During ER stress eIF2 α will be phosphorylated and thereby inhibits global protein synthesis. This adaptive mechanism prevents protein accumulation and consequent cytotoxic effects. In parallel of protein synthesis shutdown, some proteins, such as ATF4, are specifically induced. ATF4 transcription is constitutively activated in non-stressed cells, however its translation is regulated by two open reading frames (uORF)s: uORF1 is the positive element that induces ribosome scanning and reinitiation of translation at next coding regions, whereas uORF2 is an inhibitory element that negatively regulates ATF4 expression. In non-stressed cells, ribosome scanning

downstream of uORF1 initiates translation at uORF2 inducing ribosome dissociation from ATF4 mRNA, thus preventing ATF4 translation. On ER stress conditions, protein synthesis is attenuated increasing the availability of ribosomes. This allows the reinitiation translation at the ATF4 downstream coding region leading to increased ATF4 protein synthesis¹⁸. ATF4 induces expression of proapoptotic downstream target C/EBP homologous protein (CHOP) and ATF3, leading to cell death^{19,20}

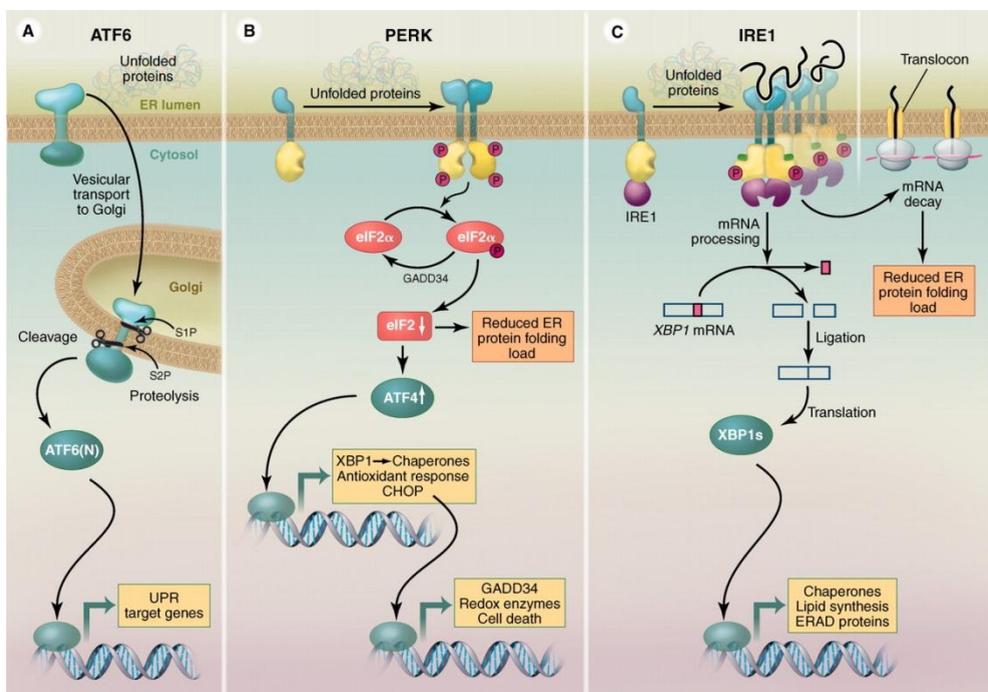


Figure 1. ER stress signaling. Three branches of unfolded protein response: ATF6, PERK and IRE-1. www.sciencemag.org

In vitro studies in INS-1E cells, primary beta cells and human islets showed increased expression of ER stress markers after cytokine, free fatty acid (FFA) and high glucose exposure and consequent beta cell damage^{11,21}. Furthermore, a study from Laybutt and colleagues observed high expression of ER stress markers in pancreatic sections of T2D patients compared to non-diabetic patients²².

ER stress-induced cell death is mediated by members of B-cell lymphoma 2 (Bcl-2) family that modulate mitochondrial permeabilization and caspase activation leading to apoptosis. Bcl-2 family is classified in three different groups: the pro-survival (Bcl-2, Bcl-XL, Myeloid cell leukemia 1, Bcl-W and A1), the pro-death (BCL2-antagonist/killer 1(Bak), BCL2-associated X protein (Bax) and BCL2-related ovarian killer (Bok)) and the BH3-only subfamily, in turn organized in activators (Bcl-2-like protein 11 (Bim), p53 upregulated modulator of apoptosis (PUMA) and truncated BH3 interacting-domain death agonist (tBID)) and sensitizers (Death protein 5 (DP5), BCL2-interacting killer (Bik), Phorbol-12-myristate-13-acetate-induced protein 1 (Noxa), Bcl-2-associated death promoter (Bad)). After cell-death stimuli the BH3-only sensitizer proteins interact with and inactivate the Bcl-2 pro-survival proteins leading to activation of BH3-only activator proteins. The unbound activators interact with Bax and Bak inducing conformational changes in these proteins and forming the mitochondrial outer membrane permeabilization (MOMP) pores. Cytochrome *c* is subsequently released from the mitochondria and binds to apoptotic protease activating factor-1

(APAF-1) forming the apoptosome complex and consequent caspase activation and apoptosis³.

Oxidative stress is another crucial mediator of beta cell failure during the development of diabetes. An imbalance between increased production of reactive oxygen and nitrogen species (ROS/RNS) and reduced levels of antioxidant defenses cause oxidative stress. It has been reported that beta cells have low expression of antioxidant defenses, such as catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx), rendering them sensitive to oxidative stress^{23,24}. In both types of diabetes increased production of mitochondrial ROS, induced by hyperglycemia, FFAs and inflammatory cytokines, cause oxidative stress and beta cell dysfunction²⁵⁻²⁷. The main source of ROS is the mitochondria. During mitochondrial respiration, molecular oxygen (O_2) plays an important role in glucose metabolism participating to oxidative phosphorylation and thus allowing ATP production. During oxidative phosphorylation oxygen is, in part, converted in superoxide anion (O_2^-) by electron transfer in the electron transport chain (ETC). O_2^- rapidly reacts with other radicals, such as NO^\bullet or iron clusters of some enzymes, inducing ROS or RNS production²⁸.

ROS and RNS induce direct oxidative damage to proteins, lipids and DNA altering enzyme activity and integrity of electron transport chain. ROS can also indirectly damage cells by activating different signaling pathways such as JNK, nuclear factor (NF)- κ B and p38 mitogen-activated protein kinase (MAPK), ultimately inducing beta cell apoptosis^{25,29}.

Loss of pancreatic beta cell mass is crucial for the development of diabetes^{30,31}. Phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) signaling pathway plays an important role in the regulation of beta cell mass^{32,33}. Several papers show that in the presence of FFAs and proinflammatory cytokines Akt phosphorylation is reduced correlating with increased apoptosis³⁴⁻³⁶. The anti-apoptotic effect of Akt is mediated by phosphorylation of different targets such as glycogen synthase kinase 3 (GSK3), forkhead box protein O1 (FOXO1) and BAD. GSK3 is a constitutively active enzyme important in the regulation of beta cell mass. Lack of Akt phosphorylation causes GSK3 activation and nuclear translocation modulating the transcription of genes related to cell proliferation and death³⁷. It has been shown that GSK3 inactivation protects beta cells against palmitate and high glucose-induced apoptosis, confirming its involvement in beta cell mass regulation³⁸. Moreover, GSK3 KD protects against ER stress-induced pancreatic beta cell apoptosis³⁹.

FOXO is a family of transcription factors also involved in cell proliferation and death. Unphosphorylated FOXO is localized into the nucleus where it regulates pro-apoptotic gene expression such as p27, Fas ligand and Bim^{40,41}. Akt phosphorylates FOXO1 allowing exclusion from the nucleus via binding to the cytoplasmic protein 14-3-3, where FOXO1 is ubiquitinated and degraded preserving cell survival⁴². Several papers show that inhibition of FOXO-1 protects pancreatic beta cells from apoptosis induced by FFAs^{43,34}. Martinez and colleagues demonstrated that expression of FOXO-1 dominant-negative allele decreases

caspase 3 and CHOP expression in MIN6 cells exposed to FFAs, demonstrating a potential role of FOXO-1 in promoting beta cell survival under lipotoxic and ER stress condition³⁵. Interestingly, FOXO-1 is also involved in beta cell protection against oxidative stress⁴⁴. Kawamori *et al* demonstrated that in pancreatic beta cells, oxidative stress induces FOXO translocation from cytoplasm-to-nucleus by JNK activation triggering apoptosis⁴⁵.

BAD is a member of Bcl-2 family which is inactivated by Akt-dependent phosphorylation. Akt phosphorylates BAD allowing its binding and inactivation by 14-3-3 proteins in the cytosol. Akt inhibition or phosphatase activation dephosphorylates BAD; consequently BAD binding to Bcl-2 or Bcl-X_L on the mitochondrial membrane leads to cell death^{46,47}. It has been demonstrated that high glucose levels led to increased pro-apoptotic BAD gene expression in human islets⁴⁸ and cytokine exposure induced BAD activation in INS-1 cells⁴⁹. Furthermore, *in vivo* studies showed that BAD mRNA levels were increased in islets isolated from mice fed high fat diet⁵⁰ and that increased beta cell survival in Zucker rats was mediated by augmented BAD phosphorylation⁵¹

1.2 The eIF2 α kinases

Phosphorylation on serine 51 of the α subunit of eIF2 is a key cellular strategy to attenuate protein synthesis in stress conditions, including ER stress, viral infection lack of amino acids or heme deficiency. eIF2 α is composed of three subunits, namely α , β and γ , binding GDP in its inactive form under physiological

conditions. Activation of protein translation requires the binding of eIF2B to eIF2 α -GDP and switching of GDP to GTP. The complex eIF2 α -GTP drives methionine-tRNA to the ribosome leading to protein synthesis. When eIF2 α is phosphorylated, eIF2(α P)-GDP complex binds more tightly the eIF2B factor preventing exchange of GDP to GTP and maintaining inactive form of eIF2 α , thus protein translation is shut off ⁵².

Four eIF2 α kinases have been identified in mammals: PERK (PKR- like ER kinase), PKR (protein kinase double-stranded RNA-dependent), GCN2 (general control non-derepressible-2) and HRI (Heme-regulated inhibitor). The catalytic domains of the eIF2 α kinases, involved in eIF2 α phosphorylation are highly homologous, whereas the stress sensing domains are specific for each member of eIF2 α kinase family ⁵³

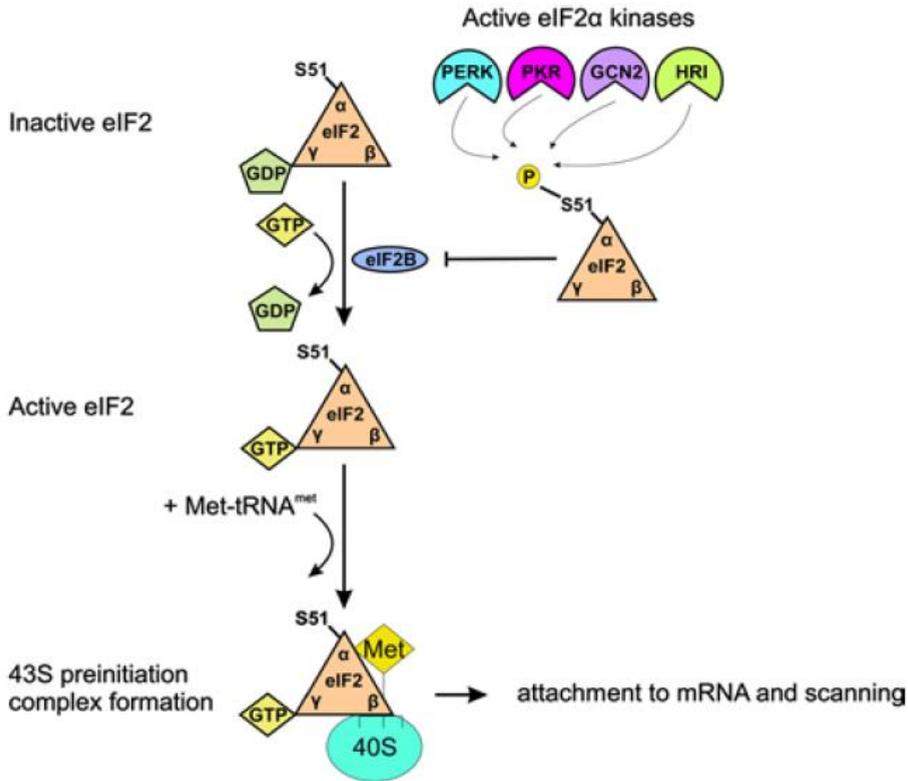


Figure 2. Schematic representation of eIF2 α status in physiological and stress conditions. eIF2 α -GDP binds eIF2B allowing the switch GDP-GTP allowing protein translation. Active PERK, PKR, GCN2 and HRI phosphorylate eIF2 α preventing eIF2B binding and thus arresting protein synthesis (Donnelly N *et al.*, *Cell Mol. Life Sci.* 2013).

As mentioned above, PERK (also known as EIF2AK3) is activated by ER stress to attenuate protein synthesis and prevent further accumulation of misfolded protein in the ER lumen. PERK is highly expressed in pancreatic beta cell where it regulates apoptosis and cell homeostasis. It has been shown that loss of PERK expression leads to the Wolcott-Rallison syndrome (WRS), a rare autosomal recessive disorder characterized by infancy-onset diabetes mellitus⁵⁴, associated

with pancreatic hypoplasia and beta cell loss⁵⁵. Zhang *et al* demonstrated that PERK knock out (KO) mice exhibited several abnormalities in fetal and neonatal beta cell mass and function, resulting in insulin secretion deficiency and decreased beta cell proliferation, thus developing permanent neonatal diabetes⁵⁶. Furthermore, several studies demonstrated that pancreatic beta cell exposure to FFAs and cytokines induces activation of PERK and its downstream targets ATF4 and CHOP^{15,57,58}.

PKR (also known as EIF2AK2) is activated by double-stranded RNA (dsRNA) produced as a result of viral infection. Activation of PKR is regulated by dsRNA binding domains (dsRBD)s contained at the N-terminal region. In unstressed conditions, dsRBDs bind the kinase domain at C-terminal region allowing to a closed structure of the enzyme and thus maintaining its inactive form. During viral replication, produced dsRNA evicts dsRBDs from kinase domain inducing PKR dimerization, autophosphorylation and thus activation. Once activated, PKR dimers are able to phosphorylate eIF2 α attenuating viral mRNA translation and triggering to apoptosis⁵⁹. Other stimuli, such as ER and oxidative stress and cytokine signaling, can lead to PKR activation⁶⁰⁻⁶³ and cause apoptosis through induction of pro-apoptotic transcription factor CHOP⁶⁴ and/or activation of NF-kB signaling⁶⁵.

GCN2 (also known as EIF2AK4) detects amino acid availability and is activated in response to amino acid starvation. Uncharged transfer RNAs (tRNAs) bind to the histidyl-tRNA synthetase (HisRS)-related domain of GCN2 inducing its activation in response of amino acid deprivation.

HRI kinase (also known as EIF2AK1) regulates protein translation in heme deficiency conditions. Heme is a hydrophobic complex of protoporphyrin ring IX and Fe^{2+} ion held in the center of the ring. Heme acts as a prosthetic group of several hemoproteins involving in important biological processes, including mitochondrial electron transport chain (i.e. cytochrome *c*), signal transduction and regulation of gene expression, gas transport and storage (i.e. myoglobin and hemoglobin) ⁶⁶. HRI plays a pivotal role in the context of hemoglobin synthesis, ensuring that α and β globin chains are synthesized in a proportionate manner of heme availability. HRI is predominantly expressed in erythroid cells where, in response to heme deficiency it is activated and phosphorylates eIF2 α decreasing globin synthesis (Figure 3). This mechanism is important to prevent accumulation of heme-free globin chains which cause ER stress and apoptosis. HRI is synthesized in the cytosol where two chaperones, heat shock protein (Hsp)-90 and Hsp-70, bind to nascent HRI protein to facilitate its folding and maintain in the inactive form. Heme binds to HRI keeping it in an inactive state. In heme deficiency conditions HRI dissociates from the Hsps leading to its autophosphorylation at multiple sites and activation ⁶⁷.

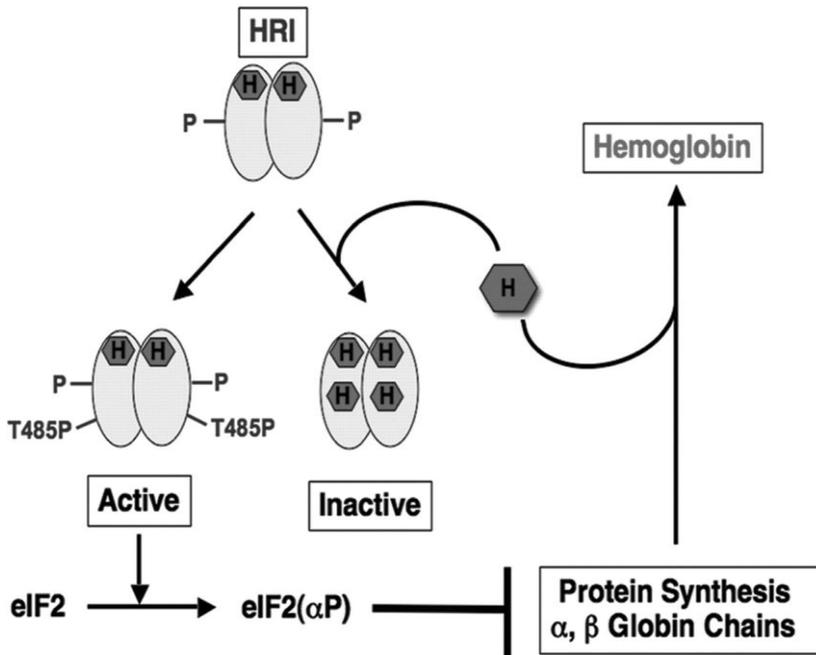


Figure 3. HRI role in hemoglobin production. HRI-HRI dimers are inactive during sufficient heme. In heme deficiency conditions, heme dissociates from HRI inducing its autophosphorylation and activation. Activated HRI leads to eIF2 α phosphorylation and consequent globin chains synthesis inhibition. (*J.J. Chen et al. Blood, 2007*)

In vivo studies demonstrated that HRI^{-/-} mice fed an iron-poor diet showed excessive accumulation and precipitation of heme-free globin chains inducing increased erythroid precursor cell death, and subsequently development of hyperchromic anemia⁵³. Furthermore, Liu S and colleagues⁶⁸ observed that HRI mediates transcription of genes involved in proliferation and apoptosis during iron/heme deficiency in erythroid cells. HRI expression has also been shown in other cell types besides erythrocytes⁶⁹. HRI has been implicated in responses to oxidative stress, heat-shock, osmotic stress and heavy metal toxicity promoting cell survival^{70,71,72,73}.

Suragani and colleagues⁷³ demonstrated that oxidative stress induced by arsenite in erythroid precursor cells, resulted in concomitant increase of ROS and HRI upregulation. The authors also observed increased HRI-induced eIF2 α phosphorylation and consequent up-regulation of ATF4 and CHOP. On the other hand, *in vitro* study on human myelogenous leukemia line (K562), demonstrated that HRI mediates cell survival during lead acetate-induced stress conditions through PI3K/Akt pathway activation. Activated HRI binds PI3K inducing phosphorylation of Akt and consequent inhibition of its downstream target BAD, resulting in decreased cell death⁷⁰ (Figure 4).

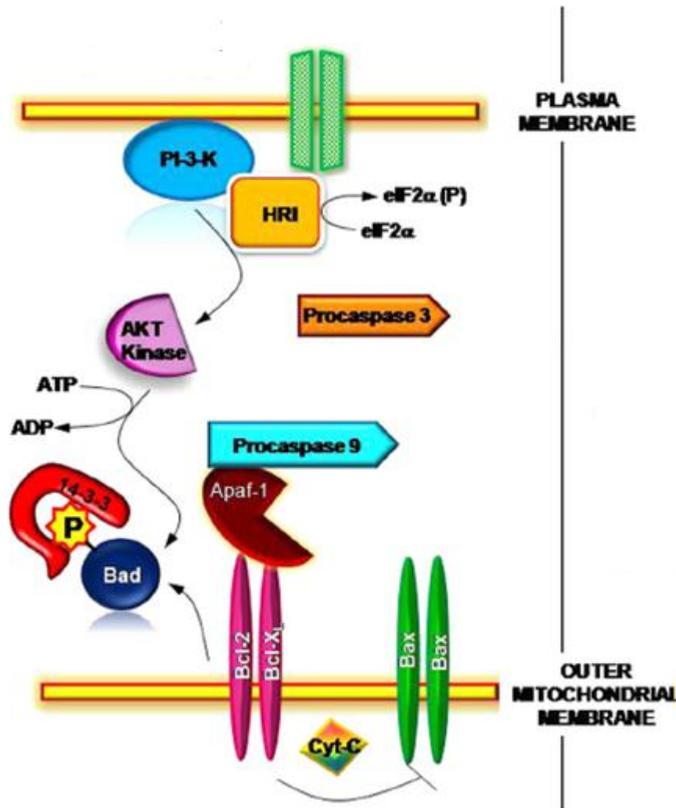


Figure 4. HRI mediates PI3K/Akt pathway. Under stress conditions, HRI binds PI3K inducing phosphorylation of Akt and consequent inhibition of BAD activity. Sequestered BAD from 14-3-3 protein, is not able to bind Bcl-2 proteins on the mitochondrial membrane resulting in apoptosis inhibition. (Figure modified from Mittal *et al.*, *Int J Biochem Cell Biol*, 2014)

Published RNA sequencing data from our group showed that in human islets⁷⁴ and primary rat beta cells⁷⁵ HRI is the most expressed eIF2 α kinase compared to other family members (Table 1). Despite its high expression levels, nothing is known regarding HRI function in beta cells.

		Human Islets	Primary rat beta cells
EIF2AK1	HRI	68,9	24
EIF2AK2	PKR	7,2	6
EIF2AK3	PERK	11,2	10
EIF2AK4	GCN2	9,2	2

Table 1. Expression levels in RPKM of the eIF2 α kinase family in human islets and primary rat beta cells.

2. Aim

Heme regulated eIF2 α kinase has been shown to be crucial to prevent accumulation of heme-free globin chains preserving erythroid cell survival in heme deficiency. HRI was also shown to mediate cell survival during stress conditions in other cell types.

RNA sequencing data from the ULB Center for Diabetes Research revealed that, in human islets and primary rat beta cells, HRI is the most expressed member of eIF2 α kinase family. Despite its high expression, the role of HRI in beta cells is unknown.

Thus, the aims of this project are to:

- Identify whether HRI modulates beta cell function;
- Study whether HRI is involved in beta cell survival in basal and diabetic conditions;
- Identify pathways involved in HRI-mediated beta cell survival.

3. Materials and methods

3.1 Culture of INS-1E and FACS-purified primary rat beta cells

Male Wistar rats (Charles River Laboratories, Brussels, Belgium) were used according to the guidelines of the Belgian Regulations for Animal Care. All experiments were approved by the local Ethical Committee. Rat islets were separated by collagenase digestion, handpicked and dispersed in single cells. Beta cells were purified by autofluorescence-activated cell sorting (FACSria, BD Bioscience, San Jose, CA, USA)⁷⁶. Preparations used in these experiments contained a purity of $95\pm 1\%$ ($n=4$). 3×10^4 beta cells *per* condition were cultured for 2 days in Ham's F-10 medium containing 10 mM glucose and 5% heat-inactivated fetal bovine serum (FBS) before further experiments.

Rat insulin-producing INS-1E cell line (kindly provided by Professor C Wollheim, Centre Medical Universitaire, Geneva, Switzerland) was cultured in RPMI-1640 + 2 mM GlutaMAX-I medium (Invitrogen) supplemented with 10 mM Hepes, 5% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 1 mM sodium pyruvate and 50 µM 2-mercaptoethanol and used at passages 60–71⁷⁷.

3.1.1 Treatments

INS-1E and primary rat beta cells were exposed to a combination of IL1 β (10 U/ml, provided by Dr. C.W. Reynolds, National Cancer Institute, Bethesda, MD)

and recombinant rat IFN γ (100 U/ml; R&D System, Oxon, UK) for 16 h to induce apoptosis mimicking T1D conditions. During cytokine exposure primary rat beta cells were maintained in normal medium in absence of serum, whereas INS-1E cells were treated in normal medium.

Palmitate (Sigma Aldrich, Schnelldorf, Germany) was dissolved in 90% ethanol, heated to 60 °C and diluted 1:100 to a final concentration of 0.5 mM in medium containing 0.75% FFA free BSA⁷⁸ for 16h to induce apoptosis mimicking T2D conditions. Deferoxamine mesylate salt (DFO, Sigma Aldrich) is an iron(III) chelator used to remove free iron from cell cultures. INS-1E cells were exposed for 24h to different concentrations of DFO (0.05 – 0.8 mM) to induce iron deficiency. Thapsigargin (Tg), a chemical ER stressor, was used at the concentration 1 μ M for 2 h as control of eIF2 α phosphorylation. For all treatments the control condition contained the same dilution of vehicle.

3.2 RNA interference

Genes were knocked down using specific siRNAs (Invitrogen) shown in table 2:

Table 2. siRNAs used to silence the expression of different genes in INS-1E and primary rat beta cells.

Gene	Species	siRNA name
HRI	Rat	H1 - Eif2ak1RSS304422
HRI	Rat	H2 - Eif2ak1RSS304424
BAD	Rat	BAD - BADRSS340056

As negative control siRNA we used 21 nucleotide duplex RNA (Qiagen, Hilden, Germany) that does not interfere with beta cell function, gene expression or viability ⁷⁹.

siRNA-lipid complexes were formed in Opti-MEM medium (Invitrogen) in a proportion of 1 μ l Lipofectamine RNAiMAX (Invitrogen) to 150 nM siRNA and incubated 20 min at room temperature. Prepared complex was added to cells in antibiotic-free medium at a final concentration 30 nM siRNA for overnight transfection. All the experiments were performed 48 hours after transfection.

3.3 mRNA Extraction and Real-time PCR

Poly(A)+RNA was isolated from INS-1E cells and primary rat beta cells using oligo-dT 25-coated polystyrene Dynabeads (DYNAL, Oslo, Norway) and reverse-transcribed to cDNA with GeneAmp RNA PCR Kit (Perkin-Elmer, Norwalk Conn, USA). cDNA was added to IQ SYBR Green Supermix (BIO-RAD, Hercules, CA, USA) and used to perform Real Time PCR on the MyiQ2 instruments (BIO-RAD).

Standards for each gene were prepared using suitable primers in a conventional PCR. A standard curve approach was used to calculate gene expression as copies per microliter and the values were corrected for the expression of the reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The specific sequences of rat primers used for real time PCR are provided in Table 3.

Table 3. Primers sequences used for quantitative Real Time PCR.

Gene	Primer forward	Primer Reverse
HRI	5'-GTGCTACGGGAAGTGAAGGT-3'	5'-GGAACTCTGTCTGTGGCTGA-3'
ATF4	5'-GTTGGTCAGTGCCTCAGACA-3'	5'-CATTCGAAACAGACGATCGA-3'
CHOP	5'- CCAGCAGAGGTCACAAGCAC-3'	5'- CGCACTGACCACTCTGTTTC-3'
XBP1t	5'-GAGCAGCAAGTGGTGGATTT-3'	5'-TCTCAATCACAAGCCCATGA-3'
BAD	5'-CCAATAACAGTCATCATGGAG-3'	5'-GTCCTCGAAAAGGGCTAAG-3'
GAPDH	5'-AGTTCAACGGCACAGTCAAG-3'	5'-TACTCAGCACCAGCATCACC-3'

3.4 Glucose-stimulated insulin secretion

INS-1E cells were cultured with RPMI-1640 without glucose supplemented with 5% FBS for 1 h. Then, cells were incubated in glucose-free modified Krebs-Ringer bicarbonate HEPES solutions⁸⁰ for 30 min. Insulin secretion was induced incubating INS-1E cells with KRBH supplemented with 1.67 or 16.7 mM glucose for 30 min. Insulin was measured by ELISA (MercoDia, Uppsala, Sweden) in cell-

free supernatants and acid-ethanol extracted cell lysates. Insulin was normalized to total protein quantified by Bradford assay.

3.5 Assessment of beta cell apoptosis

Percentage of apoptotic INS-1E cells and primary rat beta cell was determined by staining with DNA dyes Hoechst 342 (HO, 10 mg/ml) and propidium iodide (PI, 10 mg/ml) for 15 min at 37 °C. Then, cells were counted using an inverted fluorescence microscopy (excitation at 365 nm for HO and at 565 nm for PI). Live and early apoptotic cells show HO uptake (blue fluorescence). Live cells present intact nuclei while early apoptotic cells have fragmented nucleus. PI (red fluorescence) is added to discriminate necrotic or late apoptotic cells since cells are permeable to dye when they have lost nuclear membrane integrity. Necrotic cells present red intact nuclei whereas apoptotic cells show red fragmented nuclei. A minimum of 600 cells were counted in each experimental condition. Data are expressed as percentage of apoptosis or as apoptotic index. The apoptotic index was calculated against the percentage of apoptotic cells in the control condition⁸¹.

3.6 Infection with recombinant adenoviruses

INS-1E cells were infected with AdLUC (a luciferase expressing control virus), AdXBP1s (expressing mouse XBP1s⁸² and AdCMV-Akt1 (Vector biolabs, Malvern, PA) using the indicated multiplicity of infection (MOI). MOI represents the ratio of number of virus particles to number of target cells in a delimited area

(well). Adenovirus were added in normal INS-1E cells medium containing 1% FBS. After 3h of infection, medium was changed.

3.7 Western blot

Cells were washed once with ice cold phosphate-buffer saline (PBS), lysed and collected using Laemmli buffer (60 mmol/l Tris pH 6.8, 10% Glycerol, 1% SDS, 0.001% blue Bromophenol and 5% β -mercaptoethanol). Cell lysates were boiled for 5 min, separated on 9-12% SDS-PAGE gels and transferred to nitrocellulose/PVDF membranes (Hybond-ECL; Amersham Biosciences). Western blot analysis of protein expression and phosphorylation were performed using specific primary rabbit/mouse antibodies described in table 4. After incubation with secondary horseradish peroxidase-labeled anti-rabbit or anti-mouse antibody (1:10000, Jackson Immuno Research, Baltimore Pike, PA, USA), protein specific signals were detected using chemiluminescent substrate Luminol (Thermo scientific, Rockford, IL, USA) with the ChemiDoc system (Biorad). The membranes were stripped by incubation in 2% (w/v) SDS, 50 mM Tris/HCl, 150 mM NaCl, 100 mM 2-mercaptoethanol, pH 7.4, for 10 min at 55 °C in a shaking water bath to remove primary antibodies. Quantification of detected bands was performed with ImageJ software.

Table 4. Antibodies used for Western blot experiments.

Antibodies	Band size (KDa)	Dilution	Company
Phospho-eIF2α (Ser51)	38	1:1000	Cell signaling
eIF2α	38	1:1000	Cell signaling
ATF4	49	1:1000	Cell signaling
CHOP	27	1:1000	Cell signaling.
IRE-1	130	1:1000	Cell signaling
XBP1s	29/40	1:1000	Santa Cruz Biotec.
Phospho-Akt (Ser473)	60	1:10000	Cell signaling
Akt	60	1:10000	Cell signaling
Phospho-BAD (Ser136)	23	1:500	Cell signaling
Phospho-GSK3 (Ser21/9)	46/51	1:1000	Cell signaling
GSK3	46	1:1000	Cell signaling
Phospho-FOXO1 (Ser256)	82	1:1000	Cell signaling
Phospho-JNK (Thr183,Tyr185)	46/54	1:1000	Cell signaling
JNK	46/54	1:1000	Cell signaling
GAPDH	38	1:5000	Trevigen
Tubulin	55	1:20000	Sigma

3.8 Statistical analysis

Data are presented as mean \pm s.e.m. Comparisons were made by two-sided paired or ratio t-test or by ANOVA followed by paired t-test with Bonferroni correction for multiple comparisons. A p-value <0.05 was considered statistically significant.

4. Results

4.1 The role of HRI in beta cell function

Defects on insulin synthesis and secretion contribute to the development of diabetes mellitus⁸³. HRI is involved in regulating translational events phosphorylating eIF2 α . Since HRI is abundantly expressed in beta cells, we hypothesized that it may modulate insulin translation and insulin secretion in beta cells. Thus, to examine the role of HRI in beta cell function we performed glucose-stimulated insulin secretion in INS-1E cells deficient for HRI. Knock down (KD) of HRI was achieved using RNA interference with two different siRNAs, namely H1 and H2. As shown in figure 5A, HRI was efficiently knocked down by 59% \pm 8 for H1 and 66% \pm 10 for H2. HRI KD cells showed similar levels of insulin secretion in both basal and glucose-induced conditions compared to cells transfected with control siRNA (Figure 5B). The total insulin content was not affected by HRI siRNA (Figure 5C).

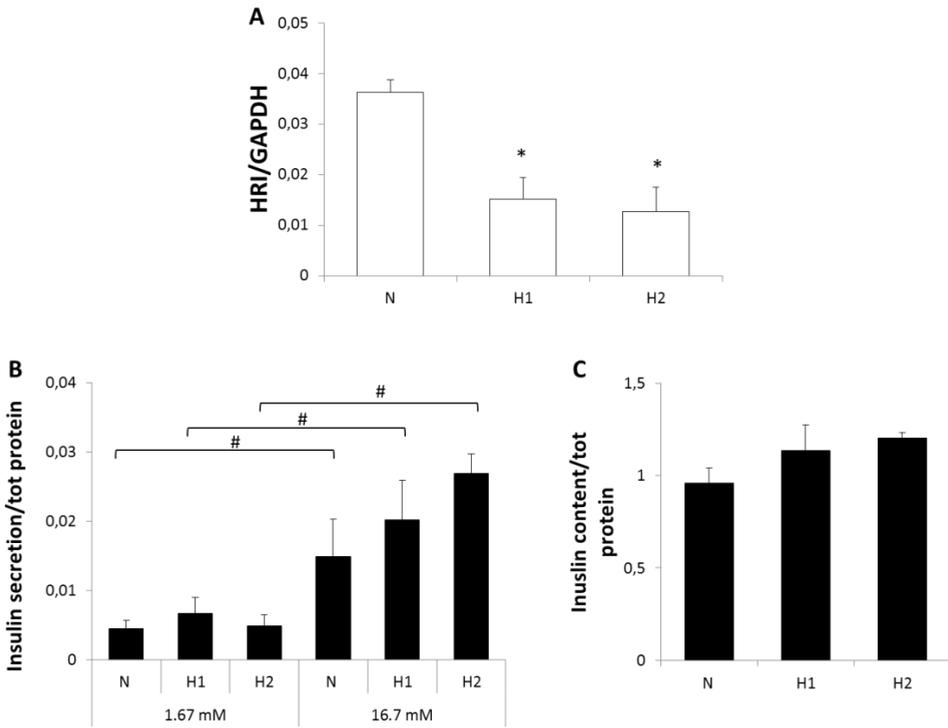


Figure 5. HRI does not affect insulin secretion. (A) HRI mRNA expression in INS-1E cells transfected with negative (control, N) or HRI (H1 or H2) siRNA; (B) ratio of secreted insulin in response to 1.67 mM or 16.7 mM glucose for 30 minutes to total protein content and (C) ratio of total insulin content to total protein content. The results represent means \pm s.e.m of 4 independent experiments. * $p < 0.05$ vs N; # $p < 0.05$ as indicated.

4.2 The role of HRI in beta cell survival

4.2.1 HRI is important for basal beta cell survival

To study the role of HRI in beta cell viability, we KD HRI with two different siRNAs in INS-1E cells and measured apoptosis. We observed that HRI depletion resulted in increased apoptosis compared to control (Figure 6A), indicating that HRI is important for basal INS-1E cell viability. On the other hand, HRI KD in primary rat beta cells did not induce apoptosis as in INS-1E cells (Figure 6C).

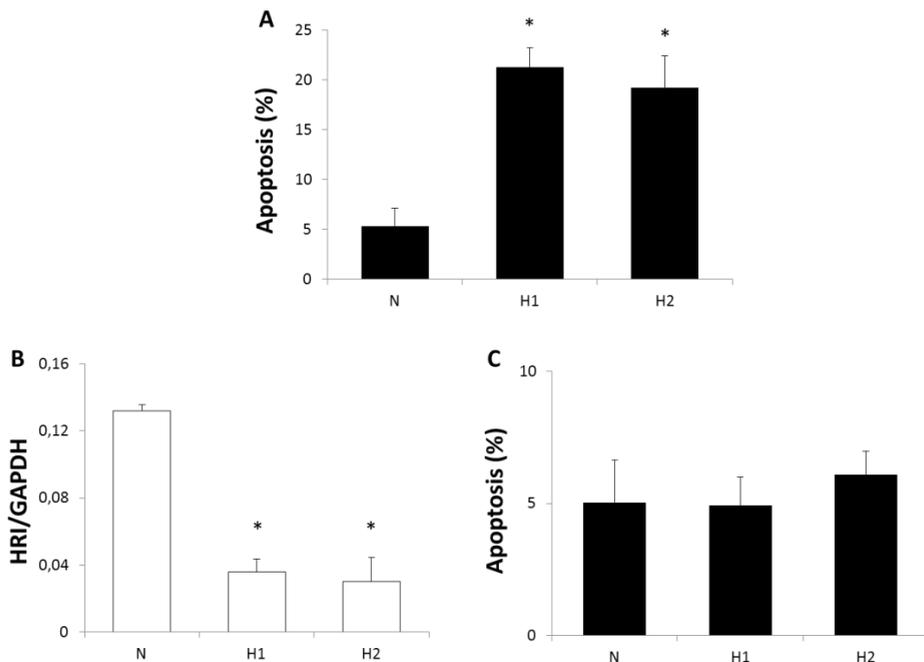


Figure 6. Effects of HRI deficiency in beta cell apoptosis. (A) Apoptosis in INS-1E cells transfected with negative (control, N) or HRI (H1 or H2) siRNA; (B) HRI mRNA

expression and (C) apoptosis in primary rat beta cells transfected with negative or two HRI (H1 or H2) siRNA. The results are the means \pm s.e.m. of 4-5 independent experiments. * $p < 0.05$ vs N.

4.2.2 HRI deficiency potentiates palmitate and cytokine-induced apoptosis

High circulating levels of FFAs have been implicated in pancreatic beta cell dysfunction and death in a phenomenon named “lipotoxicity”^{84,85}. Secretion of pro-inflammatory cytokines, such as IL1 β and IFN γ , by macrophages and T cells in islets, leads to progressive beta cell death and development of T1D⁴. In our study we used palmitate and a combination of IL1 β and IFN γ as in vitro models for T2D and T1D respectively.

HRI deficiency potentiated apoptosis induced by palmitate (Figure 7A) or cytokines (Figure 7C) as also shown as significant positive index values in Figure 7B and D respectively.

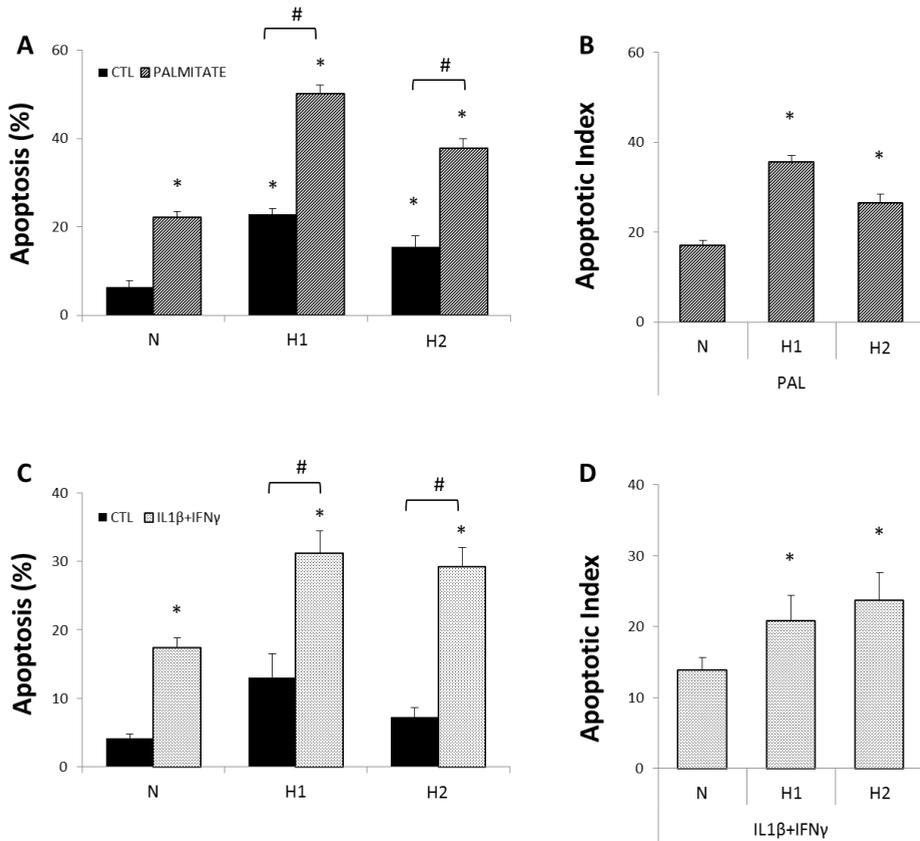


Figure 7. HRI modulates lipotoxic and inflammatory beta cell death. Apoptosis and apoptotic index in INS-1E cells transfected with negative (control, N) or two HRI (H1 or H2) siRNA and then treated with (A-B) palmitate or (C-D) cytokines for 16 hours. The results are the means \pm s.e.m. of 4 independent experiments. * $p < 0.05$ vs N CTL (untreated cells), # $p < 0.05$ as indicated.

Interestingly, our preliminary experiments showed that HRI KD in primary rat beta cells also potentiated cytokine-induced cell death compare to the control (Figure 8).

These results indicate that HRI KD sensitized beta cells to cytokine- and palmitate-

induced apoptosis. Further experiments are required to confirm the role of HRI in primary rat beta cells under lipotoxicity.

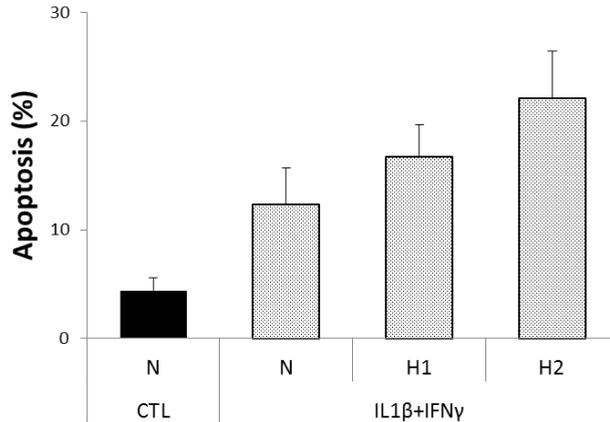


Figure 8. HRI modulates pro-inflammatory cytokine-induced primary rat beta cell death. Primary rat beta cells were transfected with negative (control, N) or two HRI (H1 or H2) siRNA and then cultured for 16 hours in the presence or absence of IL1 β + IFN γ . The results are the means \pm s.e.m. of 2 independent experiments.

4.2.3 The role of HRI in iron deficiency conditions

HRI modulates protein synthesis in iron/heme deficiency conditions to prevent globin accumulation in erythroid lineage⁸⁶. Jung and colleagues⁸⁷ reported that iron deficiency induces cytotoxic effects in INS-1E cells. To gain insights on the role of HRI during iron deprivation in beta cells, we first performed a dose-response with the iron chelator deferoxamine (DFO) to determine concentrations that induce a mild cell death. We observed increased apoptosis in a dose-dependent manner (Figure 9A) and selected two different concentrations for further experiments: 0.1 mM and 0.4 mM DFO inducing 5% and 20% of apoptosis

respectively. HRI silencing did not alter iron deficiency-induced apoptosis (Figure 9B and C), demonstrating that HRI is not involved in cell survival during iron depletion conditions.

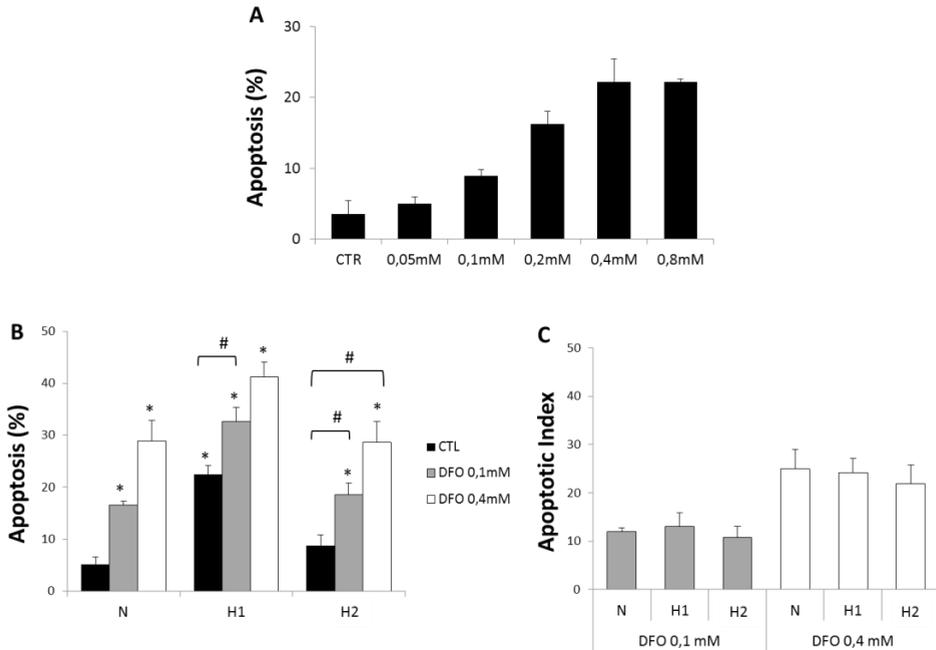


Figure 9. Iron deficiency in INS-1E cells. (A) INS-1E cells were cultured for 24 hours with the indicated concentrations of DFO in 2 independent experiments. (B) Apoptosis and (C) apoptotic index in INS-1E cells transfected with negative (control, N) or two HRI (H1 or H2) siRNA and then treated or not with DFO for 24 hours. The results are the means \pm s.e.m. of 3 independent experiments. * $p < 0.05$ vs N (untreated cells), # $p < 0.05$ as indicated.

4.3 Signaling pathways involved in HRI KD-induced apoptosis

4.3.1 eIF2 α phosphorylation does not mediate HRI KD-induced apoptosis

Since HRI is a member of the eIF2 α kinase family we performed Western blot to evaluate eIF2 α phosphorylation in HRI-depleted INS-1E cells. Unexpectedly, transfection with both HRI siRNAs resulted in a mild increase in eIF2 α phosphorylation compared to cells transfected with the negative siRNA (Figure 10A). In addition, HRI KD did not change palmitate- or proinflammatory cytokine-induced eIF2 α phosphorylation (Figure 10 B-C). As downstream targets of eIF2 α activation, we examined ATF4 and CHOP by Western blot and real-time PCR. Despite the small increase in eIF2 α phosphorylation, we did not observe significant differences in protein or mRNA levels of these targets in HRI KD cells compared to the control (Figure 10 D-E-F). These results suggest that HRI does not modulate cell survival through eIF2 α -mediated changes in ATF4 and CHOP.

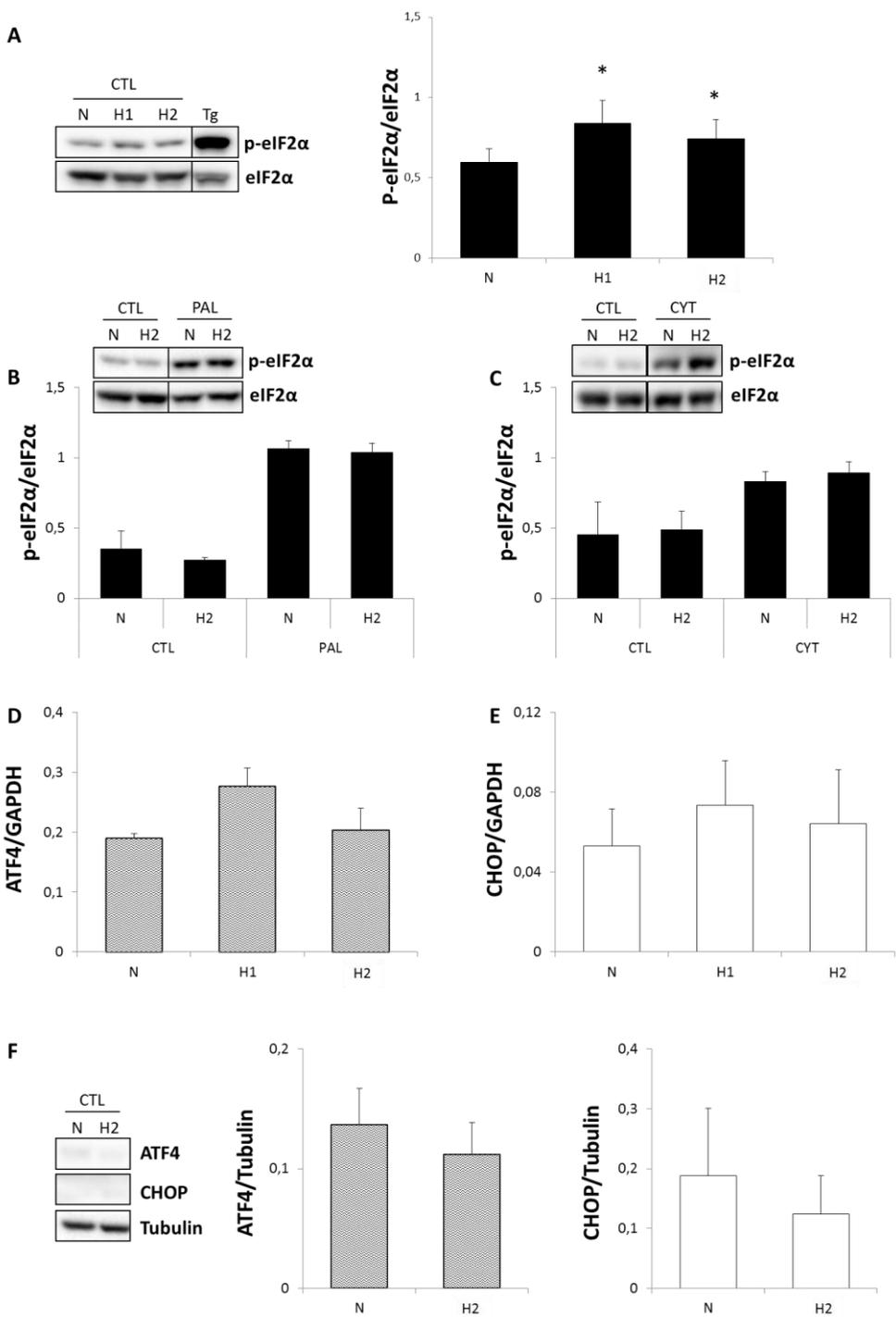


Figure 10. HRI modulates eIF2-alpha phosphorylation but not the downstream targets ATF4 and CHOP. (A) Western blots using phospho-specific eIF2 α (p-eIF2 α) antibody in INS-1E cells transfected with negative (control, N) or HRI (H1 or H2) siRNA. Total eIF2 α or tubulin were used as control for protein loading. INS-1E cells were treated for 2h with thapsigargin, an ER stress inducer, as control of eIF2 α phosphorylation. Phosphorylated eIF2 α in INS-1E cells transfected as in panel A and treated with (B) palmitate or (C) cytokines for 8 h. The mRNA levels of (D) ATF4 and (E) CHOP were analyzed by real-time PCR and normalized for the mRNA expression of the housekeeping gene GAPDH. (F) Western blot using anti-ATF4 and anti-CHOP antibodies in INS-1E cells transfected with negative (control, N) or HRI (H2) siRNA is shown. The results represent means \pm s.e.m. of 3-10 independent experiments. * $p < 0.05$ vs N.

4.3.2 HRI modulates IRE-1/XBP1s pathway

We also tested alternative branches of the ER stress response namely, IRE-1/XBP1spliced. Western blot and real-time PCR were performed to examine the expression of IRE-1, spliced XBP1 and total XBP1 in HRI KD INS-1E cells. Interestingly, we observed that IRE-1 and spliced XBP1 protein expression was downregulated by HRI depletion in INS-1E cells (Figure 11A). On the other hand, mRNA levels of total XBP1 were not changed in HRI deficiency conditions compared to the control (Figure 11B) suggesting that HRI KD induced-apoptosis could be mediated by decreased XBP1s expression and consequent low levels of ER chaperones or ERAD proteins; an important mechanism mediating cell survival in stress conditions^{17,76}.

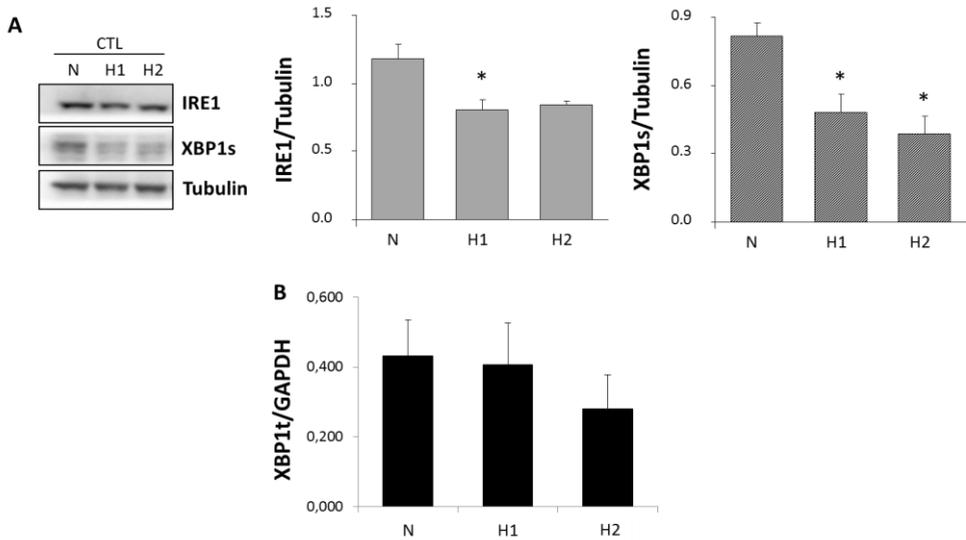


Figure 11. HRI modulates IRE-1 and XBP1s expression. (A) INS-1E cells were transfected with negative (control, N) or two HRI (H1 or H2) siRNAs, Protein expression of IRE1 and spliced XBP1 (XBP1s) are shown as well as mean optical density measurements of the Western blots of 5 independent experiments. (B) mRNA levels of total XBP1 (XBP1t) were analyzed by real-time PCR and normalized for mRNA levels of the reference gene GAPDH. The results represent means \pm s.e.m. of 5 independent experiments. * $p < 0.05$ vs N.

Next, we examined whether decreased XBP1s expression, secondary to HRI KD, caused beta cell death. For this purpose we upregulated XBP1s using an adenoviral vector in HRI-depleted INS-1E cells (Figure 12A). HRI KD-induced apoptosis was similar in cells overexpressing XBP1s or luciferase (control virus) indicating that decreased XBP1s expression is not involved in the apoptosis induced by HRI siRNA (Figure 12B).

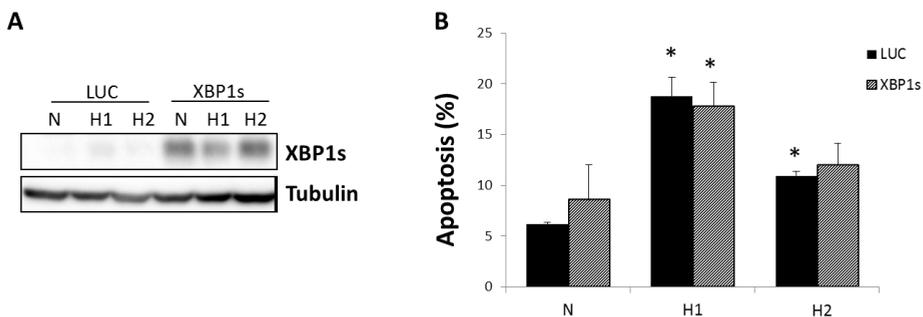


Figure 12. Decreased XBP1s expression is not involved in HRI KD-mediated beta cell death. (A) Spliced XBP1 protein expression in INS-1E cells transfected with negative (control, N) or HRI (H1 or H2) siRNA and infected with luciferase (LUC) or XBP1s-expressing adenovirus using MOI 0.5. (B) Apoptosis in INS-1E cells transfected and infected as in panel A. The results represent means \pm s.e.m. of 3 independent experiments. * $p < 0.05$ vs N transfected with LUC adenovirus.

4.3.3 HRI and PI3K pathway

Mittal and colleagues⁷⁰ demonstrated that HRI modulates cell fate during stress conditions through PI3K interaction. HRI-PI3K lead to phosphorylation of Akt and consequently the inactivation of the downstream proapoptotic target BAD. According to these data, we tested the effect of HRI KD in the PI3K signaling

pathway, evaluating phosphorylation levels of Akt and its downstream targets BAD, GSK3 and FOXO1 by Western blot. Transfection with H1 and H2 siRNA decreased phosphorylation of Akt and all its downstream targets as shown in Figure 13. These data suggest that HRI may modulate INS-1E cell survival through activation of Akt via downstream inhibition of GSK3, FOXO-1 and BAD^{35,38,76}.

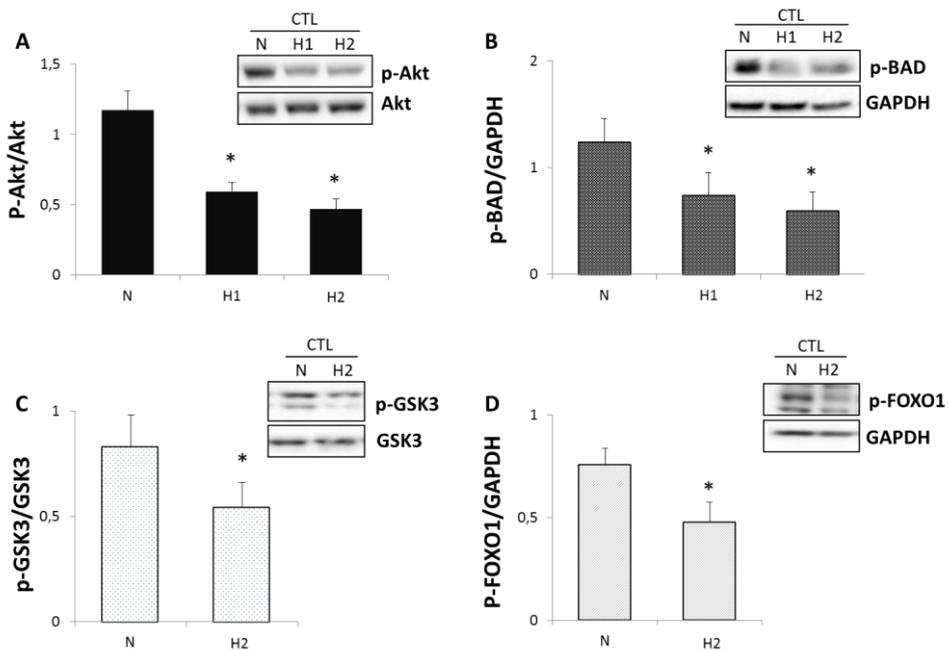


Figure 13. HRI modulates AKT signaling. INS-1E cells were transfected with negative (control, N) or two HRI (H1 or H2) siRNAs. AKT (A), BAD (B), GSK3 (C) and FOXO1 (D) protein phosphorylation was quantified by Western blots. Total Akt, GSK3 or GAPDH were used as control for protein loading. The results represent means \pm s.e.m. of 3-5 independent experiments. * $p < 0.05$ vs N

Since HRI KD decreased Akt and its downstream targets phosphorylation, we investigated whether this pathway is involved in HRI silencing-induced apoptosis.

We infected HRI KD INS-1E cells with adenovirus overexpressing a constitutively active form of Akt or luciferase (Figure 14A) and examined cell death. We observed that upregulation of the activate form of Akt partially protected INS-1E cells from HRI KD-induced apoptosis suggesting an important role for Akt (Figure 14B).

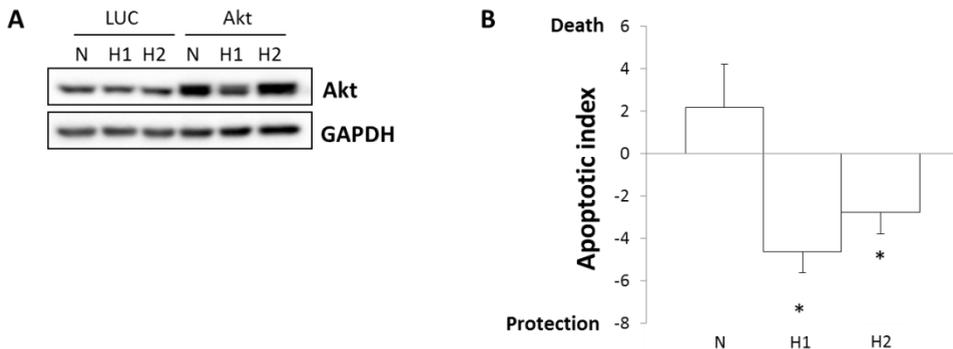


Figure 14. HRI-mediated beta cell survival is partially Akt dependent. (A) Akt protein expression in INS-1E cells transfected with negative (control, N) or two HRI (H1 or H2) siRNA and infected with luciferase (LUC) or Akt-constitutively active form adenovirus (MOI 1). (B) Apoptotic index of INS-1E transfected and infected as in panel A. The results represent means \pm s.e.m. of 3 independent experiments. * $p < 0.05$ vs N.

Based on Mittal's paper⁷⁰ and our results showing BAD dephosphorylation in HRI-KD INS-1E cells, we tested whether BAD activation (dephosphorylation) could modulate HRI deficiency-induced apoptosis. We double knocked down HRI and BAD in INS-1E cells (Figure 15 B-C) and observed that BAD silencing protected against HRI KD-dependent apoptosis (Figure 15A). These results indicate that HRI-mediated BAD inhibition is crucial for beta cell survival. Further

experiments are required to test whether GSK3 and FOXO-1 targets also play a role in HRI-dependent pancreatic beta cell survival.

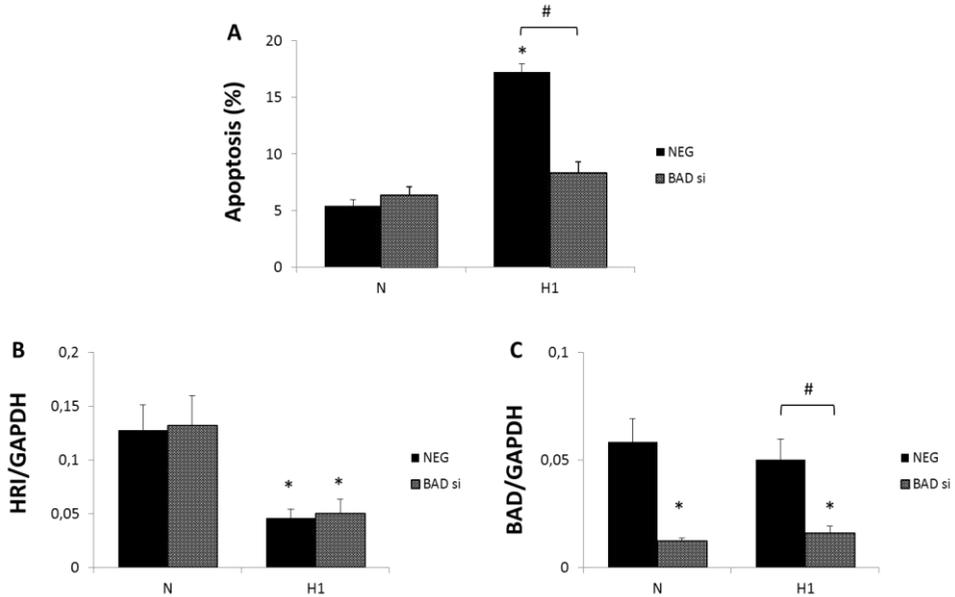


Figure 15. HRI KD-induced beta cell apoptosis is mediated by BAD. (A) Apoptosis in INS-1E cells transfected with negative (control, N), HRI (H1) and/or BAD siRNA. mRNA expression of (B) HRI and (C) BAD was analyzed by real-time PCR and normalized for mRNA levels of the reference gene GAPDH. The results represent means \pm s.e.m. of 4 independent experiments. * $p < 0.05$ vs N (black bar); # $p < 0.05$ as indicated.

4.3.4 HRI inhibits JNK activation

HRI has been shown to be activated by oxidative stress induced by arsenite, heat shock, and osmotic stress in reticulocytes⁷². Several studies showed that JNK activation is involved in apoptosis by negatively regulating Akt activity^{36,88}. Moreover, JNK inhibition protects beta cells from oxidative or ER stress and pro-

inflammatory cytokines^{24,89,90}. To determine whether HRI KD alters JNK activation, we evaluated JNK phosphorylation by Western blot in HRI-silenced INS-1E cells. We observed increased JNK activation in HRI-depleted cells compared to control (Figure 16). These results identify JNK as a putative direct/indirect target of HRI.

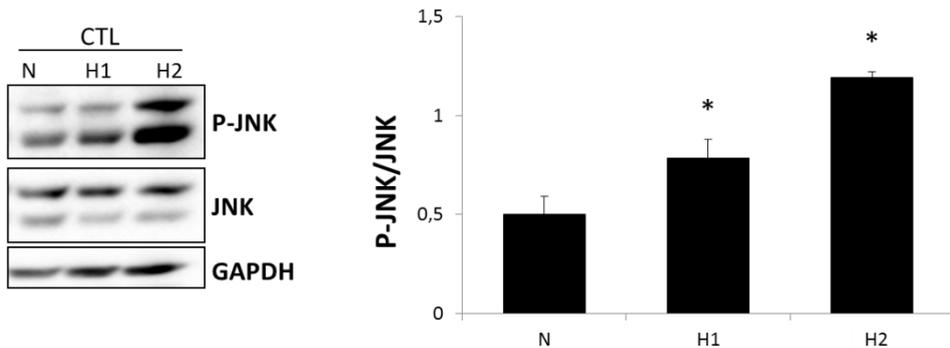


Figure 16. HRI KD induces JNK activation. INS-1E cells were transfected with negative (control, N) and two HRI siRNA (H1 or H2). Western blot using phospho-specific JNK (p-JNK) JNK antibody. Total JNK and GAPDH were used as control for protein loading. Panel on the right is the densitometry results of blots from panel on the left. The results represent means \pm s.e.m. of 3 independent experiments. * $p < 0.05$ vs N

5. Discussion

In the present study we investigated the role of HRI in pancreatic beta cells. HRI was the first identified eIF2 α kinase and it is widely conserved throughout evolution suggesting an important role of this protein. RNA sequencing data from our lab showed that in human islets⁷⁴ and primary rat beta cells⁷⁵ HRI is the most expressed eIF2 α kinase compared to the other family members. Considering the abundance of HRI and its potential role in protein synthesis we investigated the function of this eIF2 α kinase in beta cells. It has been demonstrated that PERK is required for insulin synthesis and secretion in beta cells⁵⁶. We hypothesized that HRI could also modulate insulin translation/secretion in beta cells by regulating eIF2 α phosphorylation. However, HRI deficiency did not affect glucose-stimulated insulin secretion or production.

In other cell types, HRI plays a crucial role in cell proliferation and apoptosis under stress conditions^{53,68,70,91}. In our study we found that HRI mediates beta cell survival as HRI KD resulted in increased apoptosis in INS-1E cells. On the other hand, viability of primary rat beta cells was not affected by absence of HRI. Since HRI is also important for cell proliferation, we speculate that unchanged viability in HRI-deficient primary rat beta cells could be related to the very slow proliferate capacity of these cells in culture compare to INS-1E cell line⁹². Furthermore, we observed that reduction of HRI by siRNA in INS-1E cells exposed to palmitate or the combination of IL1 β and IFN γ potentiated apoptosis. Similarly, apoptotic levels

of primary rat beta cells after cytokines exposure were increased in HRI deficiency. Further experiments are required to test whether HRI is protective in lipotoxic conditions. The protective role of HRI under basal and diabetogenic conditions suggests that as other eIF2 α kinases, HRI may work as a stress sensor in beta cells. Previous studies on different cell types, such as erythrocytes, macrophages and hepatocytes, demonstrated that HRI is modulated by heme/iron homeostasis^{53,93,94}. It has been shown that iron deficiency in INS-1E cells led to cell death through ER stress-induced CHOP and JNK phosphorylation⁸⁷. Confirming these results, we observed a dose-dependent increase in apoptosis in beta cells during iron deprivation. Different from erythrocytes, cell death was similar in the presence or absence of HRI suggesting that HRI does not modulate stress response secondary to iron deficiency. Hemochromatosis is a hereditary disorder in which patients are characterized by pathologic iron accumulation^{95,96}. Several reports have shown that iron overload mediates the development of diabetes in these patients as a consequence of increased ROS generation in the beta cells^{97,98}. A recent paper showed that pro-inflammatory cytokines led to higher iron content and ROS production in beta cells, leading to cell death⁹⁹. Liu et al. also demonstrated that lack of HRI potentiated the hemochromatosis phenotype in mice by inducing additional iron accumulation. Thus, further experiments are required to determine whether HRI is involved in beta cell iron homeostasis.

Another important question addressed in this project was the identification of cellular mechanism involved in HRI-mediated cell survival. eIF2 α kinase family

members are, in general, considered important mediators of cell survival under stress conditions, through their capacity to modulate eIF2 α phosphorylation and decrease total protein translation^{100–103}. Several papers reported that HRI modulates cell survival through eIF2 α phosphorylation and activation of its downstream targets ATF4 and CHOP under different stress stimuli such as arsenite, heat shock and osmotic stress^{53,70,72,73}. Our data however did not show differences in eIF2 α phosphorylation or its downstream targets after palmitate or proinflammatory cytokines treatment in HRI KD cells. eIF2 α phosphorylation in these conditions is mostly induced by PERK secondary to ER stress. Activation of IRE1/XBP1s and ATF6 pathways are also implicated in beta cell survival under stress conditions^{15,76,104}. We observed that IRE-1 and XBP1s expression were decreased in HRI deficiency. XBP1s overexpression did not, however, protect INS-1E cells from HRI depletion-induced apoptosis. These results suggest that HRI is not involved in the UPR in beta cells.

The PI3K/Akt signaling pathway has a crucial role to sustain beta cell mass in basal and stress conditions^{34–36}. Akt activation inhibits the proapoptotic proteins FOXO-1, GSK3 and BAD by phosphorylation. A recent report from Mittal and colleagues⁷⁰ showed that during stress, activated HRI binds to PI3K promoting Akt activation. In agreement with this work, we observed that HRI silencing decreased phosphorylation of Akt and its downstream targets. Upregulation of constitutively active Akt partially protected INS-1E cell from HRI depletion-induced apoptosis. Moreover, we found that downstream of Akt inactivation, BAD

mediates proapoptotic signaling in HRI KD cells. Experiments to test whether other proapoptotic Akt targets such as GSK3 and FOXO-1 are involved in beta cell death in HRI deficiency are ongoing.

We observed that JNK is activated by HRI siRNA suggesting that HRI may work as a possible negative regulator of JNK. Interestingly, JNK is a negative regulator of Akt and is also involved in beta cell apoptosis^{21,24,89}. More experiments are necessary to fully characterize the crosstalk between HRI-JNK-Akt and its importance for beta cell viability.

In conclusion, we showed that HRI is a crucial prosurvival factor in beta cells in both basal or diabetogenic conditions. HRI promotes XBP1 signaling, Akt/BAD activation and JNK inhibition (Figure 17). HRI represents a novel interesting target to modulate beta cell fate in diabetic conditions.

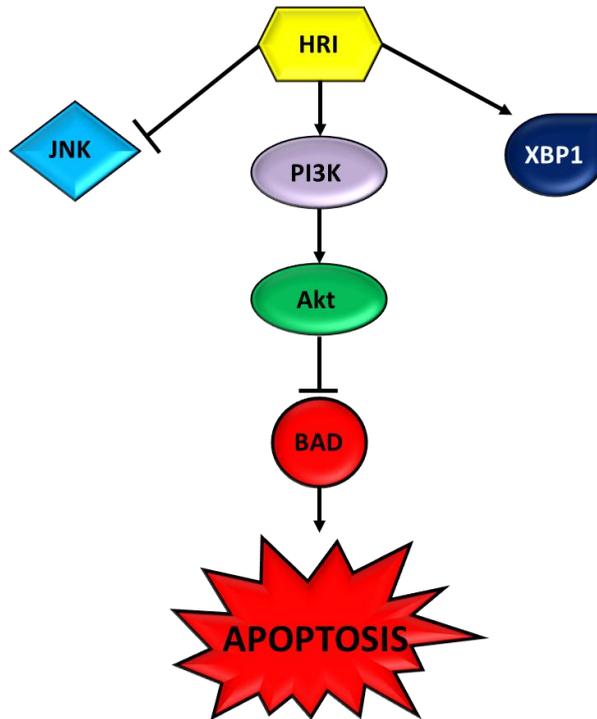


Figure 17. Schematic representation of proposed HRI mechanism. HRI inhibits JNK and activates XBP1s and Akt. Akt activation leads to BAD inhibition and thereby modulates beta cell survival.

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