The Role of ER stress and the Unfolded Protein Response in Obesity associated Type 2 Diabetes

Thesis submitted for the degree of Doctor of Philosophy

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August 2011

Abstract

Topic: The role of ER stress and the Unfolded Protein Response in obesity associated type 2 diabetes

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Pancreatic β-cell dysfunction plays a central role in the pathogenesis of type 2 diabetes. This dysfunction is characterised by secretory defects in the β-cells and the loss of β-cell mass, at least in part secondary to increased β-cell apoptosis. Although the mechanisms through which β-cell dysfunction develops are unclear, accumulating evidence suggests that elevated levels of circulating free fatty acids (FFAs) as can occur under conditions of obesity, play a role in the pathogenesis of type 2 diabetes. It has been suggested that endoplasmic reticulum (ER) stress and the resulting unfolded protein response (UPR) play a role in FFA induced β-cell dysfunction. This thesis was aimed at investigating the role of obesity induced ER stress in the development of β-cell dysfunction in type 2 diabetes.

The UPR was induced in MIN6 β-cells in response to both the saturated fatty acid (FA) palmitate, and unsaturated fatty acid oleate. Palmitate however induced a more marked ER stress response in comparison to oleate. Although both FAs induced ER stress, only palmitate evoked apoptosis in the β-cells, indicative of the differential signalling by unsaturated and saturated fatty acids. ER stress and evidence of functional adaptation was also observed in islets obtained from Zucker and Zucker diabetic fatty (ZDF) rodent models of obesity. The development of β-cell dysfunction in the progression from obesity to obesity associated type 2 diabetes in the ZDF rats was however not accompanied by a further increase in ER stress markers. This suggests that ER stress signalling does not play a significant role in the development of β-cell dysfunction.

In conclusion, the studies outlined in this thesis demonstrate that ER stress is induced in *in vitro* and *in vivo* models of β-cell lipotoxicity. It is however apparent, that ER stress does not contribute significantly to β-cell dysfunction and perhaps, only plays a small insignificant role in β-cell apoptosis in the pathogenesis of type 2 diabetes. It is hypothesised, that β-cell dysfunction develops in type 2 diabetes as a result of the inability of the β-cell to mount an additive UPR in response to ER stress.

Publications

Moore CEJ, **Omikorede O**, Gomez E; Herbert TP. PERK activation at low glucose concentration is mediated by SERCA pump inhibition and confers pre-emptive cytoprotection to pancreatic beta-cells.(*Molecular Endocrinology 25(2), 2011*)

Acknowledgements

I would like to say a very big thank you to my supervisor, Dr. Terry Herbert for your support throughout my PhD. Many thanks for your scientific expertise, insight and hours spent discussing results, analysing data and drawing up conclusions. I am also grateful for the time spent reading through and correcting my thesis. Many thanks also go to my industrial supervisor, Dr. Dave Smith, for your support and feedback especially regarding the animal model work in this thesis. I would also like to thank my committee members Dr. Gary Willars and Prof. John Challiss for your advice and feedback. My PhD was funded by the BBSRC and AstraZeneca.

Thanks go to Dr. Edith Gomez for mentoring me when I started in the lab and teaching me most of the lab skills I now possess. I am grateful for your help, advice and for making the viruses available for my project. Special thanks go to Tracy Gorman at AstraZeneca, for teaching me anything I know about RT-qPCR and being very helpful and supportive while I was at AstraZeneca. Thanks also go to Linda Härndahl, Alice Yu and Henry Brown at AstraZeneca for your help at various stages of the project. My thanks also go to the staff at the animal house at AstraZeneca who took care of the animals and made the pancreases available for my project. Thanks to past and present lab members for your friendship, and for lending a helping hand when I needed one. Thanks also go to Dr. Claire Moore for knowing where everything was and always being so very helpful, and to Jianling and Norhan for helping with my final experiments. Thanks to all the friends I have made in CPP who have made this journey easier than it may have otherwise been.

Special thanks go to my parents Prof. & Mrs Omikorede, for being so proud of me and supporting me in everything I choose to do. Thank you to my wonderful sisters Loro, Bonike and Ronike and my nephew Tomiwa, for being a constant source of distraction and making it just a little bit easier for me. I would like to say a very special thank you to Kola for your understanding, your support and your love.

Finally but most importantly, thank you to God Almighty, for making this possible.

Abbreviations

ACC	Acetyl-CoA carboxylase
ADP	Adenosine diphosphate
AMPK	AMP-activated protein kinase
ASK	Apoptosis-signal regulating kinase
ATF	Activating transcription factor
ATP	Adenosine triphosphate
BAD	BCL-xL/BCL-2 associated death promoter
BAK	BCL2 homologous antagonist killer
BAX	BCL2-associated X protein
BCL2	B cell leukemia/lymphoma
BID	BH3 interacting domain death agonist
BIM	BCL2 interacting mediator of cell death
BiP	Heavy chain binding protein
BSA	Bovine serum albumin
CALR	Calreticulin
CHOP	CCAAT/enhancer-binding protein (C/EBP) homologous protein
c-JUN	Cellular ju-nana
CNX	Calnexin
CPT1	Carnitine palmitoyl transferase-1
DAG	Diacylglycerol
ddH_2O	Double distilled water
DIO	Diet induced obese
DMEM	Dulbecco's modified Eagle's medium
dNTP	Deoxyribonucleotide triphosphate
EDEM	ER degradation-enhancing α-mannosidase-like protein

EDTA	Ethylenediaminetetraacetic acid
eIF2α	Eukaryotic translation initiation factor 2α
ER	Endoplasmic reticulum
ERAD	Endoplasmic reticulum associated degradation
Erdj4	ER DnaJ protein 4
ERO	Endoplasmic reticulum oxidoreductin
ERp	ER protein
ER-phagy	Endoplasmic reticulum based autophagy
ERSE	Endoplasmic reticulum stress element
FADH	Flavin adenine dinucleotide
FA	Fatty acid
FFA	Free fatty acid
GADD	Growth arrest and DNA damage
GCK	Glucokinase
GLS	Golgi localisation sequence
GLUT2	Glucose transporter 2
GPR40	G-protein coupled receptor 40
GRP	Glucose regulated protein
GSIS	Glucose stimulated insulin secretion
GSK	Glycogen synthase kinase
HFD	High fat diet
Hsp	Heat shock protein
HYOU1	Hypoxia upregulated protein 1
IKK	IκB kinase
INS-1	Rat insulinoma cell line
IP_3R	Inositol 1,4,5-triphosphate receptor
IRE1	Inositol requiring enzyme 1
IRS	Insulin receptor substrate

JNK	Jun N-terminal kinase
MAPK	Mitogen activated kinase
MEF	Mouse embryonic fibroblast
mg/dL	Milligrams/decilitre
MHC	Major histocompatibility complex
MIN6	Mouse insulinoma cell line
mM	Millimolar
mRNA	Messenger ribonucleic acid
mTOR	Mammalian target of rapamycin
NADH	Nicotinamide adenine dinucleotide
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NO	Nitric oxide
NRF2	Nuclear factor (erythroid-derived 2)-like 2
ORP150	Oxygen regulated protein 150
PBS	Phosphate buffered saline
PDI	Protein disulphide isomerase
PDK1	phosphatidylinositol-3,4,5-triphosphate-dependent kinase 1
PERK	Pancreatic eukaryotic initiation factor 2α kinase
PI3K	Phosphoinositide 3-kinase
PKB	Protein kinase B
PP	Protein phosphatase
PPI	Peptidyl prolyl *cis-trans* isomerase
PVDF	Polyvinylidene fluoride
ROS	Reactive oxygen species
RPM	Revolutions per minute
RT	Room temperature
RyR	Ryanodine receptor
S1P	Site 1 protease

S2P	Site 2 protease
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
Sec61	Secretory 61 protein
Ser	Serine
SERCA	Sarcoplasmic/endoplasmic reticulum Ca^{2+}
SH2	Src homology 2
SREBP	Sterol regulatory element binding proteins
SRP	Signal recognition particle
T1D	Type 1 diabetes
T2D	Type 2 Diabetes
TAD	Transcription activation domain
TAE	Tris-acetate-EDTA
TAG	Triacylglycerol
TCA	Tricarboxylic acid
Thr	Threonine
TRAF2	TNFR-associated factor 2
TRB3	Tribbles homolog 3
uORF	Upstream open reading frame
UPR	Unfolded protein response
UPRE	Unfolded protein response element
WFS	Wolfram Syndrome
WRS	Wolcott-Rallison Syndrome
XBP1	X-box binding protein 1
ZDFL	Zucker diabetic fatty lean
ZDFO	Zucker diabetic fatty obese
ZL	Zucker lean
ZO	Zucker obese

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CHAPTER 1

Chapter 1: Introduction

1.1 Background

The regulation of blood glucose levels is chiefly under the control of hormone secreting cells which exist as clusters of small glands known as the Islets of Langerhans in the pancreas. Of this cluster of cells, the β-cells in rodents make up 65-80% of cells in the islets of Langerhans and secrete insulin into the hepatic portal vein through the pancreatic veins. Both insulin and glucagon hormones function to maintain physiological glucose levels in the blood by exerting opposing effects. Glucagon secretion from α-cells is induced in the absence of dietary glucose to maintain fuel availability and blood glucose levels. While it has no effect on muscular tissue, its principal sites of action are the liver and adipose tissues. Glucagon stimulates the release of glucose into the bloodstream by causing the liver to convert stored glycogen into glucose in a process known as glycogenolysis. It also stimulates gluconeogenesis, the synthesis of glucose from amino acids and other non-carbohydrate precursors. During fasting, glucagon also stimulates lipolysis, a process which involves the breakdown of fatty acids from adipose tissue. Glycerol released during lipolysis serves as a source of carbon for gluconeogenesis while released fatty acids are used as fuel and are oxidised to CO_2 and H_2O by various tissues thereby reducing their utilisation of glucose as a fuel source. In contrast, increasing levels of glucose and/or nutrients such as free fatty acids (FFAs) in the blood, which may occur after the ingestion of food, stimulate the secretion of insulin to facilitate the metabolism and uptake of glucose by peripheral tissues. In addition to ingestion, insulin secretion may also be stimulated by high levels of circulating FFAs in the blood, which may occur as a result of obesity. Insulin is a major anabolic hormone which promotes the storage of nutrients in the body. Its effects include the conversion of glucose to glycogen for storage in the liver and muscular tissues, conversion of glucose to triacylglycerols in the liver and storage in adipose tissue, and the uptake of amino acids and synthesis of protein in muscular tissue. The opposing effects of insulin and glucagon maintain blood glucose at approximately 5mM (80-100 mg/dL) under basal conditions.

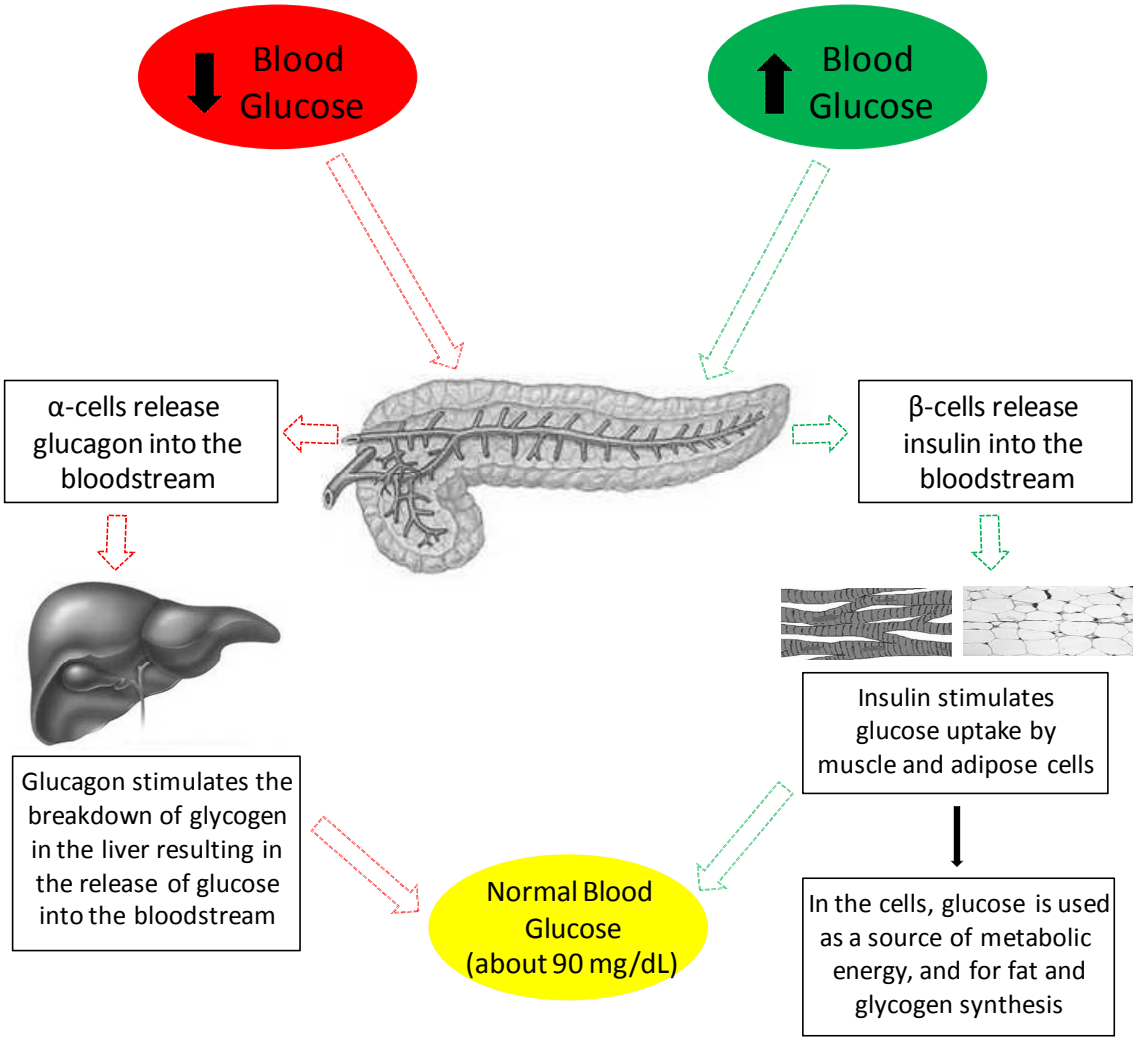


Figure 1.1 Regulation of plasma glucose homeostasis. Blood plasma glucose homeostasis is maintained by the opposing effects of insulin and glucagon. An increase in plasma glucose concentrations results in the secretion of insulin from the pancreatic β-cells, and a resultant lowering of plasma glucose concentrations. A decrease in blood plasma glucose below 'normal' levels results in the secretion of the glucagon hormone from pancreatic α-cells and a resultant increase in plasma glucose concentrations through the induction of gluconeogenesis in the liver.

1.2 The Pancreatic β-cell

The pancreas is comprised of endocrine and exocrine tissue and is located behind and below the stomach. The pancreas is predominantly made up of the exocrine glands which secrete digestive enzymes into the small intestine. By comparison, the endocrine glands make up a small portion (approximately 1-2%) of the total pancreas mass and are made up of clusters of highly vascularised secretory cells collectively known as the Islets of Langerhans. The islets of Langerhans are dispersed throughout the exocrine tissue are made up of four main cell types which secrete different hormones directly into the bloodstream. Alpha (α)-cells secrete glucagon to stimulate an increase in blood glucose, β-cells secrete insulin to stimulate the uptake of glucose from the blood, δ-cells secrete somatostatin, and PP cells secrete pancreatic polypeptide.

The pancreatic β-cells make up 65-80% of the cells in the islets of Langerhans in rodents and 50-60% of islets in humans (Cabrera, Berman et al. 2006). β-cells are chiefly responsible for the adequate synthesis and secretion of insulin in response to increases in the blood glucose levels and other nutrients such as fatty acids (McGarry, Dobbins 1999). The β-cell is exquisitely sensitive to glucose and as a result, even small changes in blood glucose levels can evoke large effects on the secretion of insulin. Circulating insulin can stimulate glucose uptake in peripheral tissue and inhibits liver glucose production amongst other functions. As mentioned above, insulin secretion from the β-cells may be stimulated both by glucose and FFAs.

1.2.1 Glucose stimulated insulin secretion (GSIS) in the pancreatic β-cell

In order to stimulate the secretion of insulin, glucose is transported into the β-cell by a non-insulin dependent glucose transporter GLUT2 in rodents and by both GLUT1 and GLUT2 in humans. Glucose is immediately phosphorylated by hexokinase IV (glucokinase) which inhibits its exit from the cell (Matschinsky 1996). In the cytosol, one phosphorylated glucose molecule (glucose-6-phosphate) is glycolytically metabolised to generate 2 molecules of pyruvate and ATP each in a process known as glycolysis. Pyruvate is transported into the mitochondria where it undergoes

conversion by oxidative decarboxylation into acetyl CoA (Henquin, Ravier et al. 2003). The oxidative decarboxylation of pyruvate is facilitated by a pyruvate dehydrogenase complex which possesses high activity within the pancreatic β-cell in comparison to other mammalian cell types (Newsholme, Keane et al. 2007). Acetyl CoA undergoes condensation with oxaloacetate in the mitochondria to generate citrate which enters into the citric acid cycle (TCA/Krebs cycle) and results in the production of NADH and $FADH_2$ (Henquin, Ravier et al. 2003). These metabolic processes which occur on the entry of glucose into the cell are geared towards enhancing the citric acid cycle and the generation of NADH and $FADH_2$ which enter into oxidative phosphorylation for the efficient production of ATP. An increase in the ATP/ADP ratio within the cell triggers the closure of ATP-sensitive K^+ channels, depolarisation of the plasma membrane and the influx of Ca^{2+} which stimulates the fusion of insulin-containing exocytotic vesicles with the plasma membrane and the secretion of insulin into the bloodstream (**Figure 1.2**). This process of K^+_{ATP}-dependent insulin secretion occurs rapidly in response to an increase in blood glucose and has been termed the 'first phase' or the 'triggering pathway' of insulin secretion.

The second phase of glucose stimulated insulin secretion (GSIS) is independent of K^+_{ATP} channel regulation and instead, augments the secretory response to the increased intracellular Ca^{2+} induced by the closure of the K^+_{ATP} channels in the first phase (Gembal, Gilon et al. 1992, Sato, Anello et al. 1999). Thus, both phases of insulin secretion are synergistic and therefore not strictly independent (Henquin 2000). Evidence for insulin secretion independent of the K^+_{ATP} channel was published in studies in which mice lacking functional K^+_{ATP} channels were still able to secrete insulin in response to glucose although defects in glucose metabolism were reported (Miki, Nagashima et al. 1998, Seghers, Nakazaki et al. 2000).

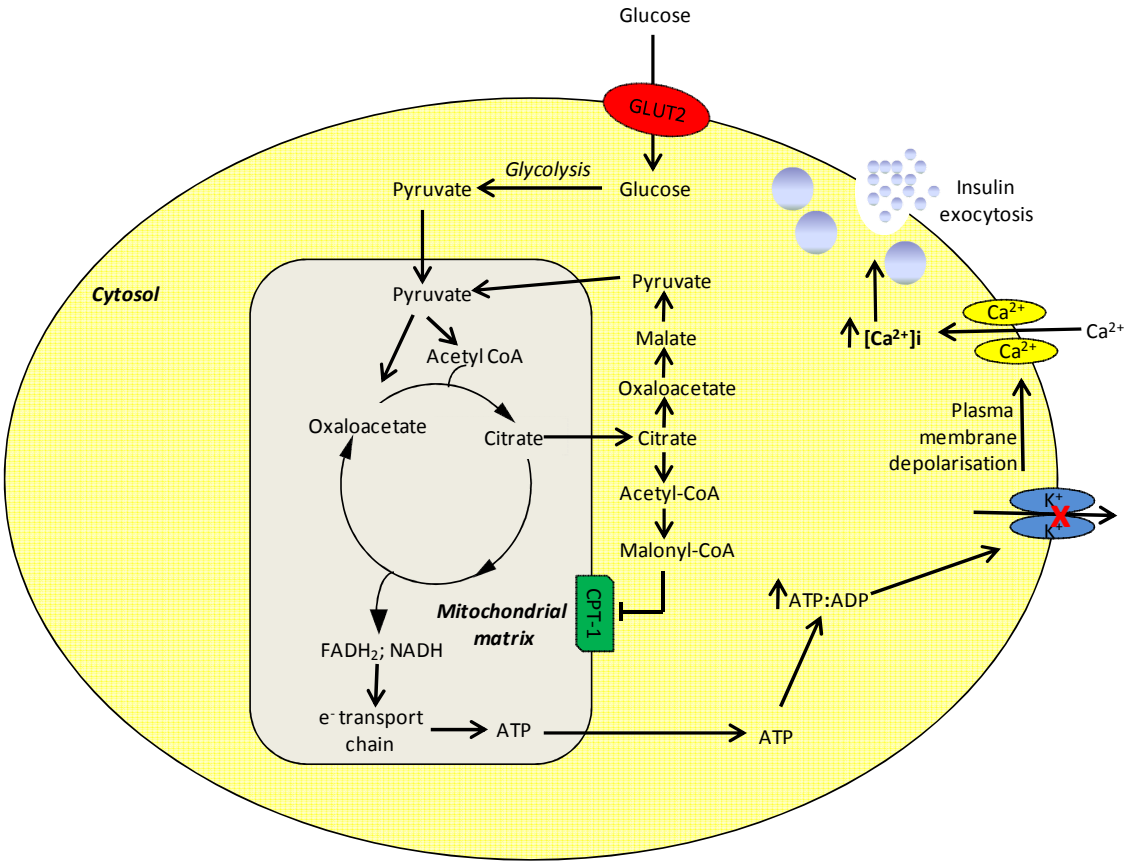


FIGURE 1.2 Model of glucose-stimulated insulin secretion in β-cells. Glucose is transported into the cell by the GLUT2 glucose transporter. Subsequent glucose metabolism results in the conversion of glucose into pyruvate which enters into the tricarboxylic (TCA) cycle. This results in the generation of reducing equivalents which are transferred into the electron (e^-) chain, the hyperpolarisation of the mitochondrial membrane, and the generation of ATP. Subsequent closure of K^+_{ATP} channels leads to the depolarisation of the cell membrane and the opening of voltage gated Ca^{2+} channels and as a result, an increase in intracellular Ca^{2+} levels. The influx of Ca^{2+} triggers insulin exocytosis from the β-cell. Adapted from (Guay, Madiraju et al. 2007).

1.2.2 Fatty acid stimulated insulin secretion in the pancreatic β-cell

Saturated long-chain free fatty acids (FFAs) have been shown to stimulate the secretion of insulin at both normal and elevated blood glucose levels (Pelkonen, Miettinen et al. 1968, Crespin, Greenough et al. 1969). In addition, endogenous fatty acid derived signalling molecules produced as a result of the lipolysis of intracellular TAG and phospholipid stores can also stimulate insulin secretion (Prentki, Joly et al. 2002). Long chain FFAs may be transported into the cell by free diffusion without the requirement for a molecular transporter (Hamilton, Kamp 1999). At normal or low glucose levels, FFAs, which are converted into acyl CoA on the outer mitochondrial membrane, are transported into the inner mitochondrial membrane by carnitine palmitoyl transferase 1 (CPT1). Inside the mitochondria, acyl CoA undergoes a series of oxidation, hydrolysis and thiolysis steps to generate NADH and $FADH_2$ which enter into oxidative phosphorylation to generate ATP (Nolan, Madiraju et al. 2006). The increase in ATP/ADP ratio in the cell results in the stimulation of insulin secretion into the bloodstream as previously described. FFAs have also been proposed to modulate insulin secretion by interacting with a membrane-bound G-protein coupled receptor GPR40 (Itoh, Kawamata et al. 2003, Alquier, Peyot et al. 2009).

The stimulation of insulin secretion by fatty acids at high glucose concentrations is mediated through a separate mechanism. At high levels of glucose, the cell possesses a high ATP/ADP ratio which induces the inhibition of AMP-activated kinase (AMPK) and the subsequent activation of acetyl CoA carboxylase (ACC). ACC catalyses the conversion of glucose derived acetyl CoA into malonyl CoA, an enzyme which inhibits CPT1 activity. A decline in the activity of CPT1 results in the inhibition of acyl CoA translocation into the mitochondria and as a result, cytosolic accumulation (Nolan, Madiraju et al. 2006). Increased cytosolic levels of acyl CoA and other FFA derivatives including phosphatidate and diacylglycerol (DAG) stimulates the elevation of intracellular Ca^{2+} and changes in the acylation states of key proteins involved in the regulation of ion channel activity and insulin exocytosis (Prentki, Joly et al. 2002). Long chain acyl CoA may also stimulate the secretion of insulin by directly facilitating the fusion of insulin-secretory granules with the β-cell plasma membrane (Prentki, Joly et

al. 2002). The stimulation of insulin secretion by FFAs in the presence of high glucose levels serves to potentiate GSIS.

1.2.3 Insulin signalling in insulin responsive tissues

To facilitate the uptake of glucose and other nutrients from the blood, insulin binds to the insulin receptor present on insulin sensitive tissues such as muscle and adipose tissue, including the β-cell. The insulin receptor is made up of two extracellular A subunits and two transmembrane B subunits which are held together by disulphide bonds. The binding of insulin to the extracellular domain of the receptor induces a conformational change which results in the autophosphorylation of several tyrosine residues present on the intracellular B subunits. The active insulin receptor phosphorylates tyrosine residues on insulin receptor substrates (IRS-1, IRS-2 and IRS-3) on their phosphotyrosine-binding (PTB) domains. Phosphorylated IRS proteins associate with the p85 regulatory subunit of the phosphoinositide 3-kinase (PI3K) through its Src homology 2 (SH2) domain to facilitate the activation of its p110 catalytic subunit. Once activated, the catalytic subunit of PI3K phosphorylates phosphatidylinositol (4,5) bisphosphate (PIP2) resulting in the formation of phosphatidylinositol-3,4,5-trisphosphate (PIP3). PIP3 formation stimulates the activity of PIP3-dependent kinase 1 (PDK1) which functions to activate the serine kinase protein kinase B (PKB/Akt). PKB in turn induces the deactivation of glycogen synthase kinase 3 (GSK-3) resulting in the activation of glycogen synthase and thus glycogen synthesis from glucose. Through this mechanism of insulin binding to its receptor and the induction of a series of biochemical reactions in the cell, excess glucose, fatty acids and amino acids in the blood are taken up into the liver, adipose and muscle tissues where they are stored in the form of glycogen, lipids and proteins respectively. Signalling downstream of the insulin receptor also stimulates several phosphorylation and dephosphorylation cascades resulting in nuclear events, ion transport and protein synthesis and degradation.

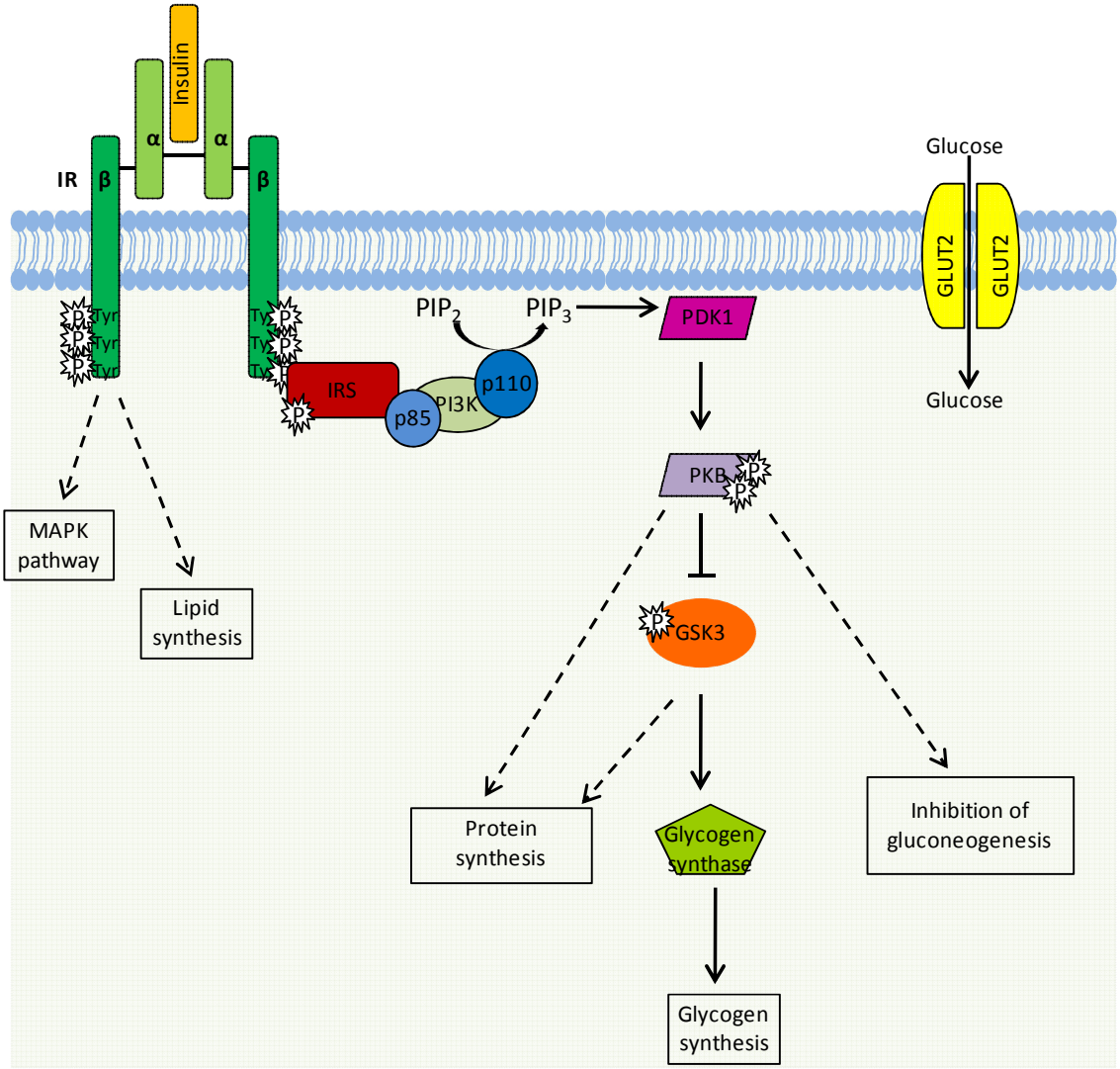


FIGURE 1.3 Insulin signalling system. The binding of insulin to its receptor results in the autophosphorylation of tyrosine residues on the intracellular domains of the insulin receptor (IR) and the subsequent phosphorylation of tyrosine residues on IRS proteins. Phosphorylation of IRS proteins results in the activation of a signalling cascade culminating in glycogen synthesis from glucose taken up into the cell through GLUT2 transport. Insulin signalling also leads to the activation of diverse signalling events which include protein synthesis, lipid synthesis and the inhibition of gluconeogenesis.

1.3 The Endoplasmic Reticulum

On nutrient induced stimulation of insulin secretion in β-cells, there is a concomitant activation of the insulin biosynthetic machinery to maintain the insulin secretory capacity of the β-cell. As a result of this tight coupling, proinsulin biosynthesis accounts for approximately 50% of the total protein synthesis in the β-cell (Schuit et al. 1988). The endoplasmic reticulum (ER) of the eukaryotic cell is a closed internal compartment which forms a continuous membrane network (Palade 1956) in the cytosol. The ER is classed as the first organelle in the secretory pathway of eukaryotic cells (Schroder 2008) with approximately a third of all cellular proteins translocated into the ER lumen (Kanapin, Batalov et al. 2003). Protein synthesis takes place at the endoplasmic reticulum which is particularly highly developed in the β-cell to facilitate the efficient synthesis of insulin to match the demand in response to constant changes in blood glucose and FFA levels. As a result of constant demand, the processes involved in the synthesis of insulin from the ER and the secretion from the β-cell are highly regulated at every step. Membrane and secretory proteins such as insulin are synthesised on the cytosolic surface of the ER and undergo conformational changes under a rigorous quality control system within the lumen of the ER before export to target organelles within the cell.

1.3.1 Protein Synthesis

The ER functions mainly as the site for membrane and secretory protein synthesis. Translation of these proteins from messenger RNA (mRNA) is facilitated by ribosomes present on the cytosolic surface of the ER (Adesnik, Lande et al. 1976, Lande, Adesnik et al. 1975). The resultant polypeptide chain possesses a signal sequence which is recognised by a cotranslationally synthesised signal recognition particle (SRP) in the cytosol (Lande, Adesnik et al. 1975, Kurzchalia, Wiedmann et al. 1986, Nagai, Oubridge et al. 2003). The associated SRP is recognised by an ER membrane localised SRP receptor which facilitates the dissociation of the SRP and targets the ribosome-polypeptide chain complex to the membrane embedded Sec61 complex (Walter, Johnson 1994, Jungnickel, Rapoport 1995). The first step in insulin synthesis is the translation of insulin mRNA as a single chain precursor known as preproinsulin. After

association with a SRP and association with an SRP receptor on the ER membrane, preproinsulin is translocated into the lumen of the ER through the Sec61 complex. On entry into the ER, the signal peptide is cleaved from preproinsulin to generate proinsulin (Orci, 1982). The Sec61 complex is a heterotrimeric complex of membrane proteins which form a pore in the membrane of the ER (Matlack, Mothes et al. 1998). The association of the ribosome-polypeptide chain complex with the Sec61 complex on the membrane of the ER results in the translocation of newly translated proteins from the cytosol into the lumen of the ER (Pilon, Romisch et al. 1998). Protein translocation into the ER lumen has also been suggested to be assisted by the luminal Hsp70 chaperone protein, BiP/GRP78, through additional gating of the Sec61 pore (Hamman, Hendershot et al. 1998).

1.3.2 Protein folding and post-translational modifications

The second major function of the ER is the folding and post–translational modification of newly synthesised polypeptide chains into their native conformation. Proteins are translocated into the ER lumen in an unfolded conformation which is characterised by higher numbers of hydrophobic patches than the native conformation (Stevens, Argon 1999). Protein folding is initially driven by the internalisation of hydrophobic amino acid residues into the core of the protein to provide a stable framework for subsequent protein folding steps (Gething, Sambrook 1992). The folding of proteins and post-translational modifications are catalysed in highly ordered steps by specific enzymes and chaperone proteins within the ER lumen. Protein post-translational modifications within the ER lumen include N-linked glycosylation and formation of disulphide bonds.

1.3.2.1 Oxidative protein folding

The formation (oxidation), breakdown (reduction) and isomerisation of disulphide bonds between cysteine residues in newly synthesised proteins is catalysed by thiol oxidoreductases (Ellgaard, Ruddock 2005). ER oxidoreductases include ERp57, ERp72, ERp5 and PDIs (protein disulphide isomerases) which are the best characterised (Mazzarella, Srinivasan et al. 1990, Ferrari, Soling 1999). PDIs are characterised by

several thioredoxin-like domains and catalytic domains with a characteristic CXXC amino acid motif (C is cysteine, X is any amino acid) containing two-redox active cysteines (Ellgaard, Ruddock 2005). Many PDIs also possess chaperone protein activity and have also been described to function as holdases for unfolded proteins within the ER lumen (Hayano, Hirose et al. 1995). Most redox-active PDIs possess two activities, an oxidase activity for the formation of disulphide bonds, and a disulphide isomerase activity (Schroder 2008). Most proteins that enter into the secretory pathway frequently require the formation of disulphide bonds for their maturation, stability and/or function (Malhotra, Kaufman 2007). Thus, newly translocated proinsulin undergoes oxidation within the ER lumen to facilitate the formation of three intramolecular disulphide bonds between the A- and B-chain components. To facilitate continued oxidase activity, PDI is re-oxidised by the flavin adenine dinucleotide (FAD)-dependent oxidases Ero1p/ERO1-Lα, ERO1-Lβ and Erv2p (Sevier, Kaiser 2002). The activity of these enzymes results in the terminal transfer of electrons to molecular oxygen resulting in the formation of reactive oxygen species (ROS). Oxidative protein folding in the ER is reported to be responsible for ~25% of all ROS generated in eukaryotic cells (Tu, Weissman 2004, Haynes, Titus et al. 2004).

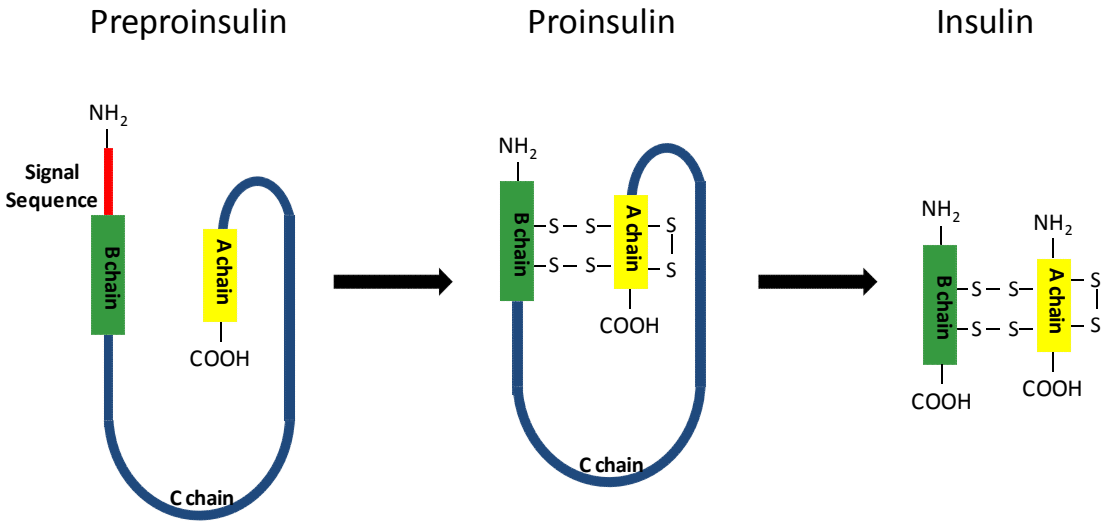


Figure 1.4 Insulin biosynthesis and conversion. Schematic diagram showing the enzymatic conversion of preproinsulin to proinsulin and then to native insulin. The insulin mRNA is translated as a single chain precursor known as preproinsulin and directed into the ER lumen by the signal sequence. In the ER, the signal sequence is cleaved off and disulphide bonds are formed, giving rise to proinsulin which is transported to the Golgi apparatus and packaged into large dense core vesicles (LCDV). The C-peptide is cleaved off giving rise to mature insulin. (Adapted from (Araki, Oyadomari et al. 2003)).

In vitro protein folding experiments suggest a quasi-stochastic mechanism that superimposes the formation and isomerisation of disulphide bonds over conformational protein folding in the ER (Welker, Wedemeyer et al. 2001). Protein folding into native conformations is catalysed by chaperone proteins which are subdivided into three separate chaperone protein systems.

1.3.2.2 HSP70 class chaperone proteins

HSP70 class ER-resident molecular chaperones such as binding protein/glucose regulated protein 78 (BiP/GRP78) (Gething 1999) and oxygen-regulated protein 150kD (ORP150/HYOU1) (Saris, Holkeri et al. 1997) constitute the first class of chaperone proteins. HSP70 class chaperones are characterised by a conserved ~44-kDa N-terminal adenosine triphosphatase (ATPase) domain and a conserved ~15-kDa C-terminal substrate binding domain (Gething 1999). These chaperones exist in adenosine

diphosphate (ADP-) and ATP-bound states and cycle through rounds of ATP hydrolysis and ADP-ATP exchange which is required for protein folding by inducing conformational changes in the unfolded proteins (Gething 1999, Hendershot, Wei et al. 1996). In an inactive state, HSP70 chaperones including BiP form stable dimeric and oligomeric complexes (Blond-Elguindi, Fourie et al. 1993, Freiden, Gaut et al. 1992). Substrate binding to HSP70 class chaperones stimulates the ATPase activity of the chaperones and the ability to cycle between an ATP- and ADP-bound state confers significantly different binding affinity for their substrate proteins (Gething 1999, Wegele, Muller et al. 2004). HSP70 class chaperones actively promote protein folding and have a higher affinity for completely unfolded or unstructured polypeptide chains in the ADP-bound form (Gething 1999). Chaperones which actively promote protein folding are referred to as chaperone foldases (Winter, Jakob 2004). The ATP hydrolysis and ADP-ATP exchange of HSP70 chaperones are stimulated by the activity of co-chaperones (Schroder 2008). DnaJ–like/HSP40 co-chaperones such as ERdj3, ERdj4, ERdj5 and Sec63p stimulate the ATPase activity of the chaperone proteins and increase the stability of the chaperone-substrate complex (Misselwitz, Staeck et al. 1998, Schroder, Kaufman 2006, Shen, Hendershot 2005, Dong, Bridges et al. 2008). The regulation of HSP70 chaperone activity through the action of co-chaperones is the basis for ATP-consumption in protein folding (Schroder 2008).

1.3.2.3 HSP90 class chaperone proteins

The HSP90 class ER-resident chaperone, GRP94 makes up the second class of chaperones involved in protein folding within the ER lumen (Argon, Simen 1999). The chaperone protein is made up of an N-terminal regulatory and nucleotide binding domain, a charged region, a central imperfect basic leucine zipper (bZIP) domain and a substrate binding and dimerisation C-terminal domain (Schroder 2008, Argon, Simen 1999, Dutta, Inouye 2000). Despite its identified ATPase activity (Dollins, Warren et al. 2007, Frey, Leskovar et al. 2007, Obermann, Sondermann et al. 1998), no co-chaperones have been shown to regulate the ATPase activity of GRP94. GRP94 has a higher affinity than other chaperone proteins for partially folded substrates. It therefore acts after BiP in the protein folding process (Argon, Simen 1999). GRP94 has

also been described as a holdase, a chaperone which binds to unfolded proteins but does not undergo substrate binding-release cycles such as the HSP70 chaperones. Holdases bind to and present the unfolded substrates to foldases, PDIs and PPIs which function to direct substrate folding (Winter, Jakob 2004). The dissociation of GRP94 with its substrate is triggered by the structural maturation of the substrate mediated by other chaperone proteins. GRP94 therefore functions as a chaperone buffer through its holdase activity under conditions of ER stress.

1.3.2.4 Lectin chaperones

The lectin chaperones make up the third arm of the protein folding machinery within the ER. These chaperones include calnexin (Bergeron, Brenner et al. 1994) and calreticulin (Bergeron, Brenner et al. 1994, Michalak, Corbett et al. 1999) and form the quality control (QC) system within the ER. The lectin chaperones specifically promote the folding of glycosylated proteins within the ER lumen by binding to monoglycosylated glycans present on the unfolded proteins (Ellgaard, Helenius 2003). Monoglycosylated glycans are produced by a series of steps which induce the addition of a hydrophilic oligosaccharide to a newly synthesised protein molecule. The oligosaccharide is initially generated on the cytoplasmic surface of the ER and is then flipped into the ER lumen through the activity of a bidirectional flippase (Hirschberg, Snider 1987). The attachment of the nascent oligosaccharide to the protein within the ER lumen is catalysed by the translocon associated enzyme, OST (oligosaccharyl transferase) which attaches the carbohydrate to the asparagines (N) amino acid in the amino-acid sequence NXS/T (asparagine-X-serine/threonine) by the formation of an N-glycosidic bond. The attached oligosaccharide is composed of $Glc_3Man_9GlcNAc_2$ in a flexible three-branched structure with the branches labelled A, B and C where Glc is glucose, Man is Mannose and GlcNac is N-acetylglucosamine. The attachment of the oligosaccharide to the protein is followed by the cleavage of glucose residues by the action of two enzymes: glucosidase I and II. Glucosidase I induces the specific cleavage of the terminal glucose residue from the A chain while glucosidase II removes the next two glucose residues from the same chain. The removal of the initial two glucose residues induces the recognition of the N-glycosylated protein by the lectin

chaperones which regulate the correct folding of most N-linked glycoproteins within the ER (Helenius, Aebi 2004). Once the correct folded protein conformation is achieved, the third glucose is cleaved from the protein by glucosidase II causing the protein to be released from the QC system and transported out of the ER (Groenendyk, Michalak 2005). The premature cleavage of the last glucose residue or misfolding of the protein results in recognition of the protein by another member of the QC system, the enzyme GT (UDP-glucose glycoprotein glucosyltransferase). This enzyme replaces the removed glucose with a new glucose residue thereby allowing the binding of the QC chaperones to facilitate protein folding (Parodi 2000). The ER QC system thus functions to prevent the export of misfolded or unfolded glycoproteins and non-glycosylated proteins from the ER (Ellgaard, Helenius 2003, Johnson, Michalak et al. 2001).

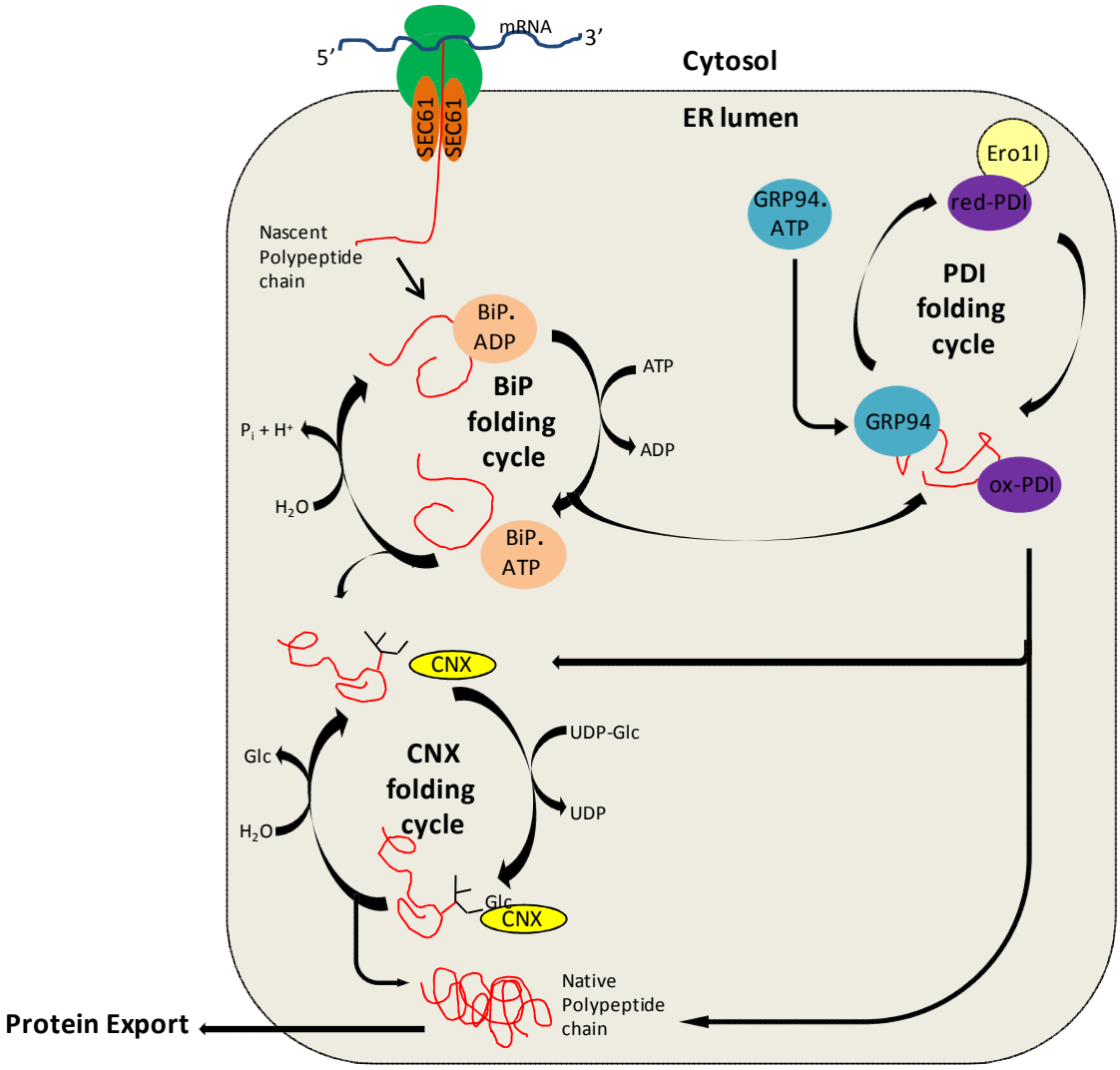


FIGURE 1.5 Chaperone protein folding system. Nascent, unfolded polypeptides enter the ER co-translationally through the Sec61 translocation complex and engage in ATP-consuming BiP.ATP-ADP hydrolysis cycles which assist the initial folding of the polypeptide chain. Partially folded proteins may be transferred to the calnexin folding cycle or to the HSP90 chaperone GRP94 which assists in protein folding in association with the chaperone activity of PDI. Folding in the calnexin cycle consumes ATP, because of the consumption of UDP-D-glucose in this cycle. When proteins are no longer recognised as being unfolded by any of the chaperone systems, and do not carry specific retention or retrieval signals, they are exported from the ER. Glc, D-glucose; P_i, inorganic phosphate. Adapted from (Schroder 2008).

1.3.3 Endoplasmic reticulum associated degradation (ERAD)

Despite the highly organised system for protein synthesis and post-translational modifications, some proteins do not achieve their native conformation within the ER lumen. This may occur as result of a mutation or lack of sufficient energy within the cell to drive the cycles of chaperone mediated protein folding (Marciniak, Ron 2006). The cell has therefore developed a mechanism for the removal of such proteins known as ER-associated degradation (ERAD).The ERAD pathway is constitutively active under basal conditions in order to protect cells against the build up of unfolded or misfolded proteins. The degradative capacity is however increased after a lag period of 30-90 min after an increase in protein synthesis, when the levels of unfolded/terminally misfolded proteins may be at their highest (Lippincott-Schwartz, Bonifacino et al. 1988). This time lag is proposed to be regulated by the activity of ER mannosidase I at least for the degradation of unfolded/misfolded glycoproteins. Mannosidase I removes mannose residues from N-linked glycans thereby making them inaccessible to the lectin chaperones and thus, marking them for degradation (de Virgilio, Kitzmuller et al. 1999, Jakob, Burda et al. 1998). Mannosidase I overexpression has been shown to enhance ERAD in HEK 293 cells (Hosokawa, Tremblay et al. 2003). In addition to mannosidase I, ER degradation-enhancing α-mannosidase-like protein (EDEM), a stress inducible but catalytically inactive homolog of mannosidase I, has also been shown to interact with misfolded glycoproteins to mediate their extraction from the ER lumen (Molinari, Calanca et al. 2003, Eriksson, Vago et al. 2004). The removal of misfolded proteins from the ER lumen requires translocation into the cytosol where such proteins are subsequently degraded. While the path(s) through which translocation occurs is not entirely clear, the Sec61 complex has been suggested to function bidirectionally for both the initial translocation of nascent polypeptides and retrotranslocation of misfolded proteins to and from the ER lumen respectively (Zhou, Schekman 1999, Plemper, Bohmler et al. 1997). A new complex made up of derlin 1, valocin-containing protein-interacting membrane protein (VIMP) which is a p97 interacting protein, and the cytosolic ATPase p97 has also been implicated in the retrotranslocation of major histocompatibility complex (MHC) class I molecules from the ER lumen (Ye, Shibata et al. 2004, Lilley, Ploegh 2004). On entry into the cytosol,

misfolded proteins initially undergo polyubiquitination and subsequent degradation by the 26S proteasome (Meusser, Hirsch et al. 2005, Gardner, Shearer et al. 2001, Hampton, Gardner et al. 1996).

In addition to ERAD, autophagy has been described as an alternative pathway for the degradation of unfolded/misfolded proteins in the ER. During autophagy, cells engulf parts of the cytoplasm and organelles and fuse with lysosomes where the engulfed particles are degraded by acid hydrolases (Yorimitsu, Klionsky 2007). The accumulation of unfolded/misfolded proteins in the secretory pathway has been reported to trigger the formation of ER sub-compartments such as Russell bodies (Valetti, Grossi et al. 1991), BiP bodies (Hobman, Zhao et al. 1998), ER-associated compartments and proliferation of stacked ER cisternae (Cox, Chapman et al. 1997). These compartments are enriched in aggregates made up of misfolded/unfolded proteins complexed with chaperone proteins such as BiP (Cox, Chapman et al. 1997, Nishikawa, Hirata et al. 1994), calnexin and calreticulin (Kamhi-Nesher, Shenkman et al. 2001).

1.3.4 Calcium storage

The ER also functions as an intracellular Ca^{2+} storage compartment in higher eukaryotes. Cellular Ca^{2+} homeostasis is especially important in the regulation of protein folding within the ER (Berridge 2002). The influx and efflux of Ca^{2+} from the ER is controlled by three ubiquitously expressed transmembrane proteins namely the ryanodine receptor (RyR) which is involved in Ca^{2+} release from the ER (Fill, Copello 2002), the inositol 1,4,5-trisphosphate receptor (IP_3R) which also releases Ca^{2+} from the ER (Nadif Kasri et al. 2002) and the sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA) 1-3 (Vangheluwe, Raeymaekers et al. 2005). The influx of Ca^{2+} from the cytoplasm into the ER is primarily controlled by the pumping action of the SERCA pumps (Schroder 2008). The SERCA pumps maintain a free Ca^{2+} concentration of approximately 1-2mM in the ER by pumping Ca^{2+} against a Ca^{2+} concentration gradient resulting in ATP hydrolysis (Vangheluwe, Raeymaekers et al. 2005). The SERCA pump can also operate in the reverse manner resulting in ATP synthesis. The ability of the ER to store and release Ca^{2+} confers an important role in the activation of a wide range of cellular processes including organogenesis, transcriptional activity, stress responses

and apoptosis (Berridge 2002). ER Ca^{2+} homeostasis also controls transcriptional and translational cascades which regulate the chaperones responsible for protein folding within the ER (Groenendyk, Michalak 2005). One of such is the lectin chaperone, calreticulin. Calreticulin has been determined to bind Ca^{2+} (Johnson, Michalak et al. 2001) and as a result, has been discovered to be involved in several processes which include cellular Ca^{2+} homeostasis such as the uptake of Ca^{2+} into the ER by the SERCA (Camacho, Lechleiter 1995), Ca^{2+} storage within the ER (Nakamura, Zuppini et al. 2001) and Ca^{2+} release from the ER (Mesaeli, Nakamura et al. 1999). The regulation of Ca^{2+} within the ER is also important for the efficient secretion of insulin from the β-cell.

1.3.5 Lipid and sterol biosynthesis

Finally, the ER is also involved in the biosynthesis of unsaturated fatty acids, sterols and phospholipids (Kent 1995, Chang, Chang et al. 2006). The synthesis of cholesterol and fatty acids is regulated by a family of type II transmembrane transcription factors known as sterol regulatory element binding proteins (SREBPs) resident on the ER membrane (Chang, Chang et al. 2006). SREBP-1C mainly regulates fatty acid synthesis while SREBP-2 regulates cholesterol synthesis. When cholesterol levels are high, SREBPs are localised to the ER membrane in a complex with the cytosolic domain of another ER membrane protein known as SREBP cleavage-activating protein (SCAP). SCAP possesses a transmembrane sterol-sensing domain with which it binds to an ER membrane protein, INSIG (Bengoechea-Alonso, Ericsson 2007). Under conditions of high cholesterol levels, cholesterol is esterified in the ER membrane with unsaturated FAs (Miyazaki, Ntambi 2003) by acyl CoA: cholesterol acyltransferase 1 (ACAT1) (Chang, Chang et al. 2006). Cholesterol esters are therefore stored in the cell as lipid droplets (Chang, Chang et al. 2006). A depletion of cholesterol or unsaturated FA levels results in the dissociation of the interaction between SCAP and INSIG which in turn facilitates the translocation of the SREBP-SCAP complex to the Golgi complex within the cell (Ye, Rawson et al. 2000). Free INSIG is ubiquitinated and degraded by the 26S proteasome (Bengoechea-Alonso, Ericsson 2007); while SREBP undergoes proteolytic cleavage by the Golgi resident site-1 and site -2 proteases to generate a functional SREBP transcription factor (Bengoechea-Alonso, Ericsson 2007, Horton, Goldstein et al.

2002). SREBP is translocated into the nucleus where it induces the transcription of genes involved in the biosynthesis of cholesterol, triacylglycerols and phospholipids, saturated and unsaturated fatty acids.

1.4 ER stress

Perturbation of the major functions of the ER such as protein folding, Ca^{2+} storage or the synthesis of lipids and sterols results in the accumulation of unfolded proteins within the ER lumen (Schroder 2008). Depletion of ER Ca^{2+} results in impaired protein folding (Lodish, Kong et al. 1992), retention of unfolded proteins in the ER lumen, and a subsequent disruption of ER to Golgi transport and chaperone function (Lodish, Kong 1990; Suzuki, Bonifacino et al. 1991). Other conditions under which unfolded proteins may accumulate in the ER include an abnormal increase in the synthesis of secretory proteins, expression of misfolded proteins, DNA damage, viral infections and nutrient deprivation (Schröder, Kaufman 2005). When the ER client load exceeds the protein folding capacity as a result of misfolded protein accumulation, ER stress is induced. In the case of insulin, mutations in the insulin gene or the failure of proinsulin to achieve the correct conformations may result in its accumulation in the ER lumen and ER stress in the β-cell. To protect against ER stress, the eukaryotic cell possesses a well conserved and elaborate signalling pathway termed the Unfolded Protein Response (UPR). The UPR functions as a multifaceted strategy to protect the integrity of the ER and the associated functionality of the secretory pathway.

1.4.1 Sensing ER stress

The detection of ER stress and the subsequent activation of the UPR is initiated by three distinct ER transmembrane sensors; inositol-requiring protein-1 (IRE1), protein kinase RNA (PKR)-like ER kinase (PERK) and activating transcription factor-6 (ATF6). IRE1 and PERK are type 1 transmembrane proteins with ER luminal stress sensing domains which regulate their protein kinase activity by functioning as ER-stress regulated di- and oligomerisation domains (Shamu, Walter 1996, Liu, Schröder et al. 2000). This functional luminal domain is highly conserved with a small but significant homology in IRE1 and PERK (Liu, Schröder et al. 2000). ATF6, a type II transmembrane

bZIP transcription factor, also possesses an ER luminal domain which serves as an ER-stress regulated retention motif at the ER membrane (Chen, Shen et al. 2002). Under unstressed conditions, the luminal ER stress sensing domains are associated with the ER chaperone BiP/GRP78, an interaction which retains the transducer proteins in an inactive conformation (Bertolotti, Zhang et al. 2000, Liu, Xu et al. 2003). Activation of the UPR transducer proteins in response to ER stress has been suggested to occur via two possible mechanisms as outlined below.

1.4.1.1 The competition model of ER stress detection

Under ER stress conditions induced by the accumulation of unfolded/misfolded proteins, it is proposed that BiP/GRP78 may preferentially bind to the accumulated proteins thereby dissociating with the transducer proteins and allowing their activation in a model described as the competition model (Bertolotti, Zhang et al. 2000). Consistent with this model are reports in which an increase in the expression of secretory proteins resulted in the induction of BiP expression (Dorner, Wasley et al. 1989, Watowich, Morimoto et al. 1991) and the increased efficiency of BiP induction by slowly folding proteins which formed longer associations with BiP than rapidly folding proteins (Watowich, Morimoto et al. 1991, Gething, McCammon et al. 1986).The dissociation of BiP/GRP78 from ATF6 under ER stress conditions also coincides with the release of the transcription factor from the ER to the Golgi apparatus (Shen, Chen et al. 2002).

1.4.1.2 The ligand binding model of ER stress detection

An alternative model for ER stress detection suggests that the dissociation of BiP from the stress sensor domains may not be the sole mechanism for the detection of unfolded proteins and activation of the transducers. The deletion of the BiP binding domain in yeast Ire1p did not affect the ER stress-sensing and signalling ability of Ire1p (Kimata, Oikawa et al. 2004). In another study, an alpha helical groove structurally similar to the peptide binding domains of major histocompatibility complexes (MHCs) was identified in yeast Ire1p (Credle, Finer-Moore et al. 2005). The authors of this study suggested that direct binding of unfolded proteins occurred in this groove as

mutation of side chains facing into the groove resulted in reduced UPR response (Credle, Finer-Moore et al. 2005). A study by Zhou et al., however suggested that the corresponding groove in human IRE1 is too narrow and of the wrong orientation to facilitate an interaction with unfolded proteins (Zhou, Liu et al. 2006).

Irrespective of the mode of ER stress detection, the induction of the UPR through the activation of the transducer proteins results in the attenuation of global protein synthesis within the cell to reduce the protein load within the ER, a paradoxical increase in the translation of a specific set of mRNAs, and the transcriptional upregulation of specific genes involved in protein folding and ERAD in an attempt to alleviate ER stress. The upregulation of transcription factors in response to ER stress results in the activation of different subsets of UPR genes which modulate diverse and temporally distinct responses to ER stress. These signals are transmitted by diverse signalling pathways activated by the three proximal ER stress sensor proteins.

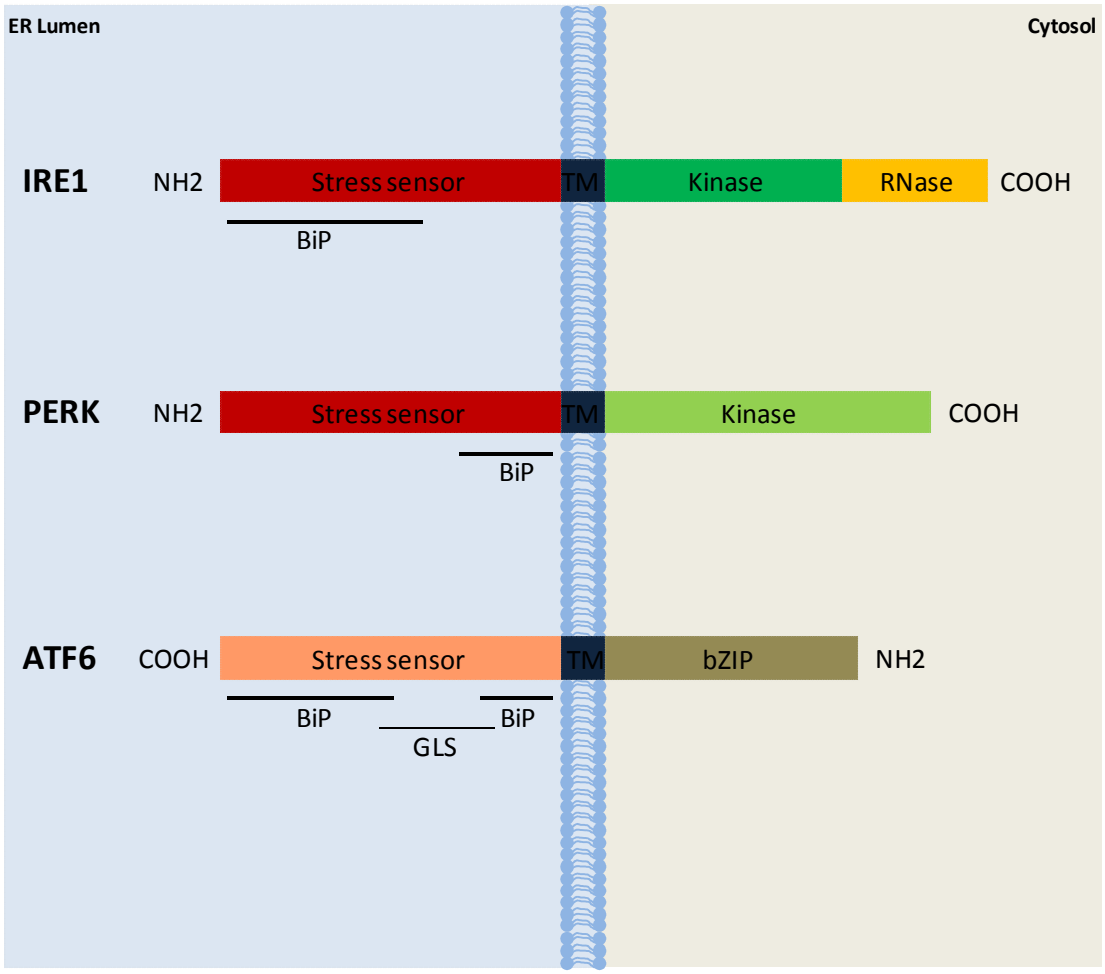


Figure 1.6 Schematic representations of the three ER stress transducer proteins: IRE1, PERK and ATF6. Transducer proteins are kept in an inactive conformation under basal conditions by their association with BiP via the BiP binding regions shown in bold lines. ATF6 possesses a Golgi localisation sequence (GLS), shown in faint line. Stress sensor domains are localised within the lumen of the ER, while the serine/threonine kinase domains of IRE1 and PERK are localised in the cytosol. IRE1 possesses an additional RNase domain with endoribonuclease activity. ATF6 possesses a basic leucine zipper domain on the cytosolic domain. COOH: carboxyl terminus; NH2: amino terminus; TM: transmembrane domain. Adapted from (Kohno 2007).

1.5 The Unfolded Protein Response

1.5.1 IRE1 signalling

IRE1 is a bifunctional type I transmembrane serine/threonine (Ser/Thr) protein kinase and site-specific endonuclease (RNAse) (Mori, Ma et al. 1993, Sidrauski, Walter 1997) and is highly conserved with known homologues in plants (Koizumi, Matrinez et al. 2001), *C. elegans* (Shen, Ellis et al. 2001)*, Drosophila* (Plongthongkum, Kullawong et al. 2007) and mammals (Liu, Schröder et al. 2000, Wang, Harding et al. 1998). IRE1 was first identified as Ire1p in yeast (Mori, Ma et al. 1993, Welihinda, Kaufman 1996) and has been found to occur in two forms in mammalian cells: IRE1α which is ubiquitously expressed and IRE1β which is expressed only in intestinal epithelial cells (Wang, Harding et al. 1998, Tirasophon, Welihinda et al. 1998). The ER luminal N-terminal domain functions both as a stress sensor and dimerisation domain (Liu, Schröder et al. 2000, Zhou, Liu et al. 2006) while the cytosolic domain contains protein kinase and endoribonuclease activity (Shamu, Walter 1996, Sidrauski, Walter 1997). Under basal conditions, the transducer protein exists as an inactive monomer on the ER membrane and is retained at the ER by a luminal interaction with BiP/GRP78 (Bertolotti, Zhang et al. 2000). Detection of ER stress, which may occur through previously described mechanisms, results in the dissociation of BiP/GRP78 from the transducer protein thereby allowing for its activation (Liu, Schröder et al. 2000, Bertolotti, Zhang et al. 2000). IRE1 activation occurs as a result of spontaneous homodimerisation of the ER luminal domains and the subsequent induction of the kinase and RNase activity through the autophosphorylation of the cytosolic domains (Liu, Xu et al. 2003). Activated IRE1 therefore functions both as a kinase and an endoribonuclease under ER stress conditions.

1.5.1.1 IRE1 induced XBP1 splicing

Ire1p functions as the sole ER stress sensor in the yeast *Saccharomyces cerevisiae*. Ire1p dimerisation in response to ER stress results in the activation of the endonuclease activity in the cytosolic domain, which induces the unconventional splicing of a 252-nucleotide intron from mRNA encoding the bZIP transcription factor,

Hac1p (Sidrauski, Walter 1997, Gonzalez, Sidrauski et al. 1999, Kawahara, Yanagi et al. 1998). The *HAC1* mRNA is constitutively expressed but only becomes spliced in response to ER stress (Cox, Walter 1996, Kawahara, Yanagi et al. 1997). Ire1p mediated cleavage of the intron at two specific sites on the 1.4-kb precursor *HAC1* mRNA (Sidrauski, Walter 1997) yields exon termini which are joined by the tRNA ligase Rlg1p (Sidrauski, Cox et al. 1996) to yield a 1.2-kb mature *HAC1* mRNA (Kawahara, Yanagi et al. 1998). As a result of unconventional splicing, a frameshift in *HAC1* mRNA results in the translation of the Hac1p C-terminal domain which functions as a potent transcription factor with a transcription-activation domain (Kawahara, Yanagi et al. 1998, Cox, Walter 1996). The transcription factor binds to promoter UPR elements (UPREs) within the nucleus to induce the transcriptional upregulation of >380 genes which make up >5% of all yeast genes (Travers, Patil et al. 2000). The upregulated genes encode proteins which function to alleviate ER stress within the cell.

In metazoans, IRE1 is activated in response to ER stress through the same mechanism of BiP/GRP78 dissociation, homodimerisation of the luminal domains and autophosphorylation of the cytosolic domains as occurs in yeast (Tirasophon, Welihinda et al. 1998). Activated metazoan IRE1 also possesses endonuclease activity and therefore induces the unconventional splicing of the X-box binding protein 1 (*XBP1)* mRNA (Calfon, Zeng et al. 2002) in a manner reminiscent of *HAC1* mRNA splicing in yeast. Endonucleolytic splicing of the *XBP1* mRNA results in the removal of a 26-nucleotide intron and the introduction of a frameshift in the *XBP1* mRNA (Yoshida, Matsui et al. 2001). The resultant spliced *XBP1* mRNA encodes a potent transcription factor with an alternative C-terminus known as spliced XBP1 (XBP1s), a functional homologue to Hac1p in yeast (Yoshida, Matsui et al. 2001, Lee, Tirasophon et al. 2002). XBP1s is translocated into the nucleus where it binds to the cis-acting ER stress response elements (ERSE) in cooperation with a constitutively expressed transcription factor nuclear factor Y (NF-Y), to activate the transcription of a subset of UPR target genes which include ER chaperones and folding factors (Lee, Iwakoshi et al. 2003), and factors involved in ER membrane biogenesis (Sriburi, Jackowski et al. 2004). XBP1 also binds to the unfolded protein response element (UPRE) independent of NF-Y to induce

the specific transcription of ERAD factors such as EDEM (Travers, Patil et al. 2000, Lee, Iwakoshi et al. 2003, Friedlander, Jarosch et al. 2000).

1.5.1.2 IRE1 induced degradation of a subset of nascent mRNAs

In addition to the splicing of XBP1, activated IRE1 also functions to degrade ER-targeted mRNAs in an XBP1 independent manner thereby decreasing client protein load within the ER (Hollien, Weissman 2006, Pirot, Naamane et al. 2007). In addition to ER targeted mRNAs, activated IRE1 has also been reported to induce the cleavage of the 26S ribosomal RNA subunit (Iwawaki, Hosoda et al. 2001) thereby playing a role in translational repression in response to ER stress.

The activation of IRE1 in response to ER stress, the downstream upregulation of the UPR target genes (Travers, Patil et al. 2000, Lee, Iwakoshi et al. 2003) and the degradation of mRNAs encoding ER targeted proteins are thus adaptive mechanisms developed by eukaryotic cells to alleviate ER stress within the cell. The unspliced form of XBP1 (XBP1us), an inactive transcription factor, is also translated in stressed cells and has been suggested to act as a negative feedback regulator of IRE1 signalling by binding to and targeting XBP1s for degradation (Yoshida, Oku et al. 2006).

1.5.2 PERK signalling

The protein kinase-like ER kinase (PERK) is a type 1 ER transmembrane protein which functions as the second transducer of the UPR and is highly expressed in professional secretory cells in which ER stress may be anticipated (Kincaid, Cooper 2007, Shi, Vattem et al. 1998, Harding, Zhang et al. 1999). PERK exists as a monomer at the ER membrane and is kept in an inactive conformation through its association with the ER resident chaperone protein, BiP/GRP78 (Bertolotti, Zhang et al. 2000). The ER luminal sensor domain of the PERK protein bears homology with the IRE1p luminal domain in yeast and both domains have been shown to be functionally interchangeable (Liu, Schröder et al. 2000). ER stress as a result of protein accumulation, induces dissociation of BiP/GRP78 from PERK thus permitting dimerisation and trans-autophosphorylation of the cytosolic domains (Bertolotti, Zhang et al. 2000). PERK autophosphorylation results in the activation of the cytosolic kinase domain and the

subsequent phosphorylation of specific PERK substrates within the cell (Marciniak, Garcia-Bonilla et al. 2006).

1.5.2.1 PERK phosphorylation of eIF2α

Activation of the cytosolic kinase domain within PERK primarily results in the phosphorylation of a serine residue in position 51 (Ser-51) on the α-subunit of the eukaryotic translation initiation factor-2 (eIF2α) (Harding, Zhang et al. 1999). eIF2 is a heterotrimeric GTP-binding protein which initiates protein translation by recruiting the initiator methionyl tRNA (Met-tRNAi) to the mRNA via the 40S ribosomal subunit. The initiation of translation results in GTP hydrolysis and the release of an inactive eIF2-GDP complex. Regeneration of the active eIF2-GTP complex requires the activity of eIF2B, a heteropentamer which functions as a guanine-nucleotide exchange factor (GEF) for eIF2α (Gomez, Mohammad et al. 2002). The phosphorylated form of eIF2α (p-eIF2α) acts as a competitive inhibitor of eIF2B binding to the inactive eIF2-GDP complex thus blocking eIF2 recycling and inhibiting translation in the process (Proud 2007). PERK induced eIF2α phosphorylation therefore results in the inhibition of the global translation rate within the cell thereby reducing the protein client load within the ER lumen in an attempt to alleviate ER stress (Harding, Zhang et al. 1999, Marciniak, Garcia-Bonilla et al. 2006). In spite of the inhibition of global protein translation as a result of PERK mediated eIF2α phosphorylation, the activating transcription factor 4 (ATF4) mRNA is known to undergo a paradoxical translational upregulation under conditions of ER stress (Harding, Novoa et al. 2000b, Lu, Harding et al. 2004). The ATF4 mRNA escapes translational repression by encoding two upstream open reading frames (uORFs) in its 5' untranslated region (UTR), of which the second ORF is out of frame and overlaps with the ATF4 start codon (Vattem, Wek 2004). Under basal conditions, ribosomes scanning downstream of the first uORF (uORF1) reinitiate at the second uORF (uORF2) thereby missing the ATF4 start codon and thus, preventing ATF4 translation. ER stress conditions however, favour the reinitiation of translation at the ATF4 start codon resulting in the upregulation of ATF4 expression (Marciniak, Ron 2006, Rasheva, Domingos 2009). ATF4 is subsequently translocated into the nucleus where it induces the transcriptional upregulation of genes encoding

proteins involved in amino acid import, glutathione biosynthesis and resistance to oxidative stress (Heather, Yuhong et al. 2003). Thus, the translational upregulation of ATF4 functions to upregulate the expression of UPR target genes which would otherwise be repressed as a result of eIF2α phosphorylation. ATF4 also induces the expression of pro-apoptotic genes including the CCAAT/enhancer binding protein (C/EBP) homologous protein (CHOP) and ATF3 (Pirot, Ortis et al. 2007, Jiang, Wek et al. 2004). Growth arrest and damage inducible gene (GADD) 34 is also induced by ATF4 and functions in a negative feedback loop to induce recovery from translational repression (Novoa, Zeng et al. 2001, Connor, Weiser et al. 2001). GADD34 relieves global translational attenuation in the cell by forming a complex with the catalytic subunit of the Ser/Thr protein phosphatase (PP) 1c thereby directing its activity to the dephosphorylation of eIF2α (Connor, Weiser et al. 2001). In addition to GADD34 directed dephosphorylation of eIF2α, a constitutive repressor of eIF2α phosphorylation (*CreP*) also inhibits eIF2α phosphorylation in mammalian cells (Jousse, Oyadomari et al. 2003). CreP is constitutively expressed while the expression of GADD34 is significantly induced under conditions of ER stress (Jousse, Oyadomari et al. 2003). Modulation of PERK function has also been suggested to be mediated by the co-chaperone protein $p58^{IPK}$ which binds to the kinase domain of PERK to inhibit its activity (Yan, Frank et al. 2002, van Huizen, Martindale et al. 2003). A subsequent study however determined that $p58^{IPK}$ functions mainly as a chaperone protein due to its predominant localisation in the ER lumen (Rutkowski, Kang et al. 2007). Thus, the stress induced expression of GADD34 downstream of ATF4 appears to be central to the reversal of translational attenuation in the UPR (Novoa, Zeng et al. 2001, Brush, Weiser et al. 2003, Ma, Hendershot 2003).

Global translational repression in response to ER stress is also induced by a similar mechanism in yeast. In response to stress conditions such as amino acid starvation, the kinase Gcn2p phosphorylates eIF2α thereby inhibiting protein synthesis and inducing the upregulation of Gcn4p, a homologue of ATF4 (Dever, Feng et al. 1992, Natarajan, Meyer et al. 2001). Similar to the mechanism of ATF4 upregulation, the expression of GCN4 is induced under stress conditions as a result of the presence of short ORFs within the GCN4 mRNA (Patil, Li et al. 2004). The expression of Gcn4p in association

with its activator Gcn2p, and Hac1p which is expressed as a consequence of ER stress induced IRE1 activation are all required for the upregulation of the majority of UPR target genes under stress conditions in yeast (Natarajan, Meyer et al. 2001, Patil, Li et al. 2004).

In addition to ER stress, eIF2α may also be phosphorylated in response to other cellular stresses. eIF2α phosphorylation may be induced by the activation of four kinases which respond to distinctive stress signals within the cell. The eIF2α kinases possess strong similarity in their catalytic domains but possess distinct regulatory domains which confer specificity on the type of stress they respond to (Dever, Dar et al. 2006). As outlined above, PERK is activated in response to ER stress. The protein kinase repressor (PKR) is activated by double stranded RNA produced during viral infection and induces translational repression and apoptosis in virally infected cells (de Haro, Mendez et al. 1996). The activation of general control nonrepressed 2 (GCN2), another eIF2α kinase, is cytoprotective and occurs in response to the presence of uncharged tRNAs to adapt cells to amino acid deprivation (Natarajan, Meyer et al. 2001, Zhang, McGrath et al. 2002b). The heme-regulated eIF2α kinase (HRI) is activated in response to stress induced by the presence of free globins in iron-deficient vertebrates (Han, Yu et al. 2001). Although the upstream stress signals that activate the eIF2α kinases are distinct, the phosphorylation of eIF2α on serine 51 and the subsequent downstream effects are common to all the stress pathways resulting in the term integrated stress response (ISR) (Harding, Calfon et al. 2002, Ron 2002).

1.5.2.2 PERK phosphorylation of Nrf2

The activation of PERK in response to ER stress also leads to the phosphorylation of nuclear factor (erythroid-derived 2)-like 2 (Nrf2), the second known substrate of PERK (Cullinan, Zhang et al. 2003). Nrf2 is a cap 'n' collar bZIP transcription factor which is maintained in inactive cytoplasmic complexes through its association with a cytoskeletal anchor, Keap1 (Moi, Chan et al. 1994, Itoh, Wakabayashi et al. 1999). PERK dependent Nrf2 phosphorylation results in the dissociation of Nrf2 from the cytoplasmic complex and induces the nuclear import of the transcription factor (Cullinan, Zhang et al. 2003). Nrf2 induces the transcription of genes containing the

antioxidant response element (ARE) as a heterodimer with other bZIP transcription factors such as ATF4 (Heather, Yuhong et al. 2003, He, Gong et al. 2001), c-JUN, JUN-B and JUN-D (Venugopal 1998). Indeed, oxidative stress has been reported to induce the dissociation of the Nrf2-Keap1 complex via an uncharacterised mechanism (Itoh, Wakabayashi et al. 1999). The phosphorylation and nuclear translocation of Nrf2 is reportedly independent of eIF2α phosphorylation and is believed to be a protective response to ER stress signals as the deletion of the transcription factor was found to be detrimental to cell survival (Cullinan, Diehl 2004).

Thus, ER stress induced PERK activation results in the propagation of downstream signals which aim to repress global translation and induce the upregulation of factors which function to alleviate ER stress within the ER lumen thereby protecting the cell.

1.5.3 ATF6 signalling

The activating transcription factor 6 (ATF6) is the third transducer of ER stress signals and is ubiquitously expressed in metazoans and higher eukaryotes (Haze, Okada et al. 2001). ATF6 is a 90-kDa type II transmembrane protein with an ER luminal sensor domain which interacts with the chaperone protein BiP/GRP78 to facilitate ER retention (Chen, Shen et al. 2002). It is suggested that the disulphide and glycosylation status of ATF6 also mediates retention of the protein at the ER (Hong, Luo et al. 2004, Nadanaka, Okada et al. 2007). Indeed, the glycosylated form of ATF6 interacts with the lectin chaperone calreticulin on the ER luminal sensor domain thereby retaining the transcription factor at the ER (Hong, Luo et al. 2004). The cytosolic domain of ATF6 contains a basic-leucine zipper motif (bZIP) (**Figure 1.6**). Under ER stress conditions, ATF6 undergoes dissociation from BiP/GRP78 as previously described resulting in the unveiling of Golgi localisation signals (GLS) on the luminal domain of the protein (Shen, Chen et al. 2002). The association of ATF6 with calreticulin is also weakened under ER stress conditions as underglycosylated forms of ATF6 which may occur under ER stress conditions do not interact with calreticulin (Hong, Luo et al. 2004). Dissociation from interactions with chaperone proteins within the ER lumen results in release of ATF6 from the ER and translocation to the Golgi apparatus. At the Golgi, ATF6 is sequentially cleaved by Golgi resident site-1 protease (S1P) and S2P, resulting in the release of the

cytosolic domain of ATF6 as a functional 50-kDa transcription factor (Ye, Rawson et al. 2000, Shen, Prywes 2004). The potent transcription factor is translocated into the nucleus where it binds to the ER stress response element (ERSE) CCAAT(N)9CCACG (Yoshida, Haze et al. 1998) in genes encoding a wide range of ER chaperone proteins including lectin and HSP-70 class chaperones thereby increasing the protein folding capacity within the ER lumen (Yoshida, Haze et al. 1998, Okada, Yoshida et al. 2002). ATF6 has been shown to induce chaperone protein expression in cooperation with XBP1s, the transcription factor expressed downstream of IRE1 signalling (Yamamoto, Sato et al. 2007). ATF6 also induces the expression of XBP1 mRNA thereby supplying more substrate for IRE1 induced XBP1 splicing (Lee, Tirasophon et al. 2002). The activation of ATF6 in response to ER stress is therefore a protective signalling pathway designed to alleviate ER stress through the upregulation of chaperones and components of the ERAD machinery.

Following the discovery of ATF6, several other tissue specific ER transmembrane proteins which undergo similar translocation to the Golgi and cleavage by S1 and S2 proteases under ER stress conditions have been identified. These include OASIS which is expressed in astrocytes (Kondo, Murakami et al. 2005), Tisp40, a spermatid specific bZIP transcription factor (Nagamori, Yabuta et al. 2005) and CREBH which is activated to induce an inflammatory response (Zhang, Shen et al. 2006). While the exact roles played by these proteins have not been fully elucidated, OASIS has been implicated in ER stress signalling in astrocytes (Kondo, Murakami et al. 2005) while CREBH has been shown to undergo translocation and proteolysis during ER stress in the liver (Zhang, Shen et al. 2006).

The induction of multiple UPR signals through the activation of the three ER stress transducers serves to restore homeostatic balance by the reduction of protein load and the upregulation of folding capacity within the ER lumen. The initial response to ER stress is the global attenuation of translation mediated by PERK phosphorylation of eIF2α. This signal immediately relieves the ER lumen of the entry of more client protein load. Following PERK activation, activated ATF6 induces the transcription of ER

chaperone proteins and foldases thereby increasing protein folding capacity in the ER. The upregulation of chaperone proteins in response to IRE1 signalling is believed to be delayed in comparison to ATF6 as the production of the active XBP1 transcription factor requires splicing and translation (Yoshida, Matsui et al. 2001). Activation of UPR signalling in response to ER stress also serves to upregulate the ERAD machinery. The ERAD machinery is constitutively active in healthy cells to facilitate the removal of terminally misfolded/unfolded proteins within the ER lumen (Travers, Patil et al. 2000, Friedlander, Jarosch et al. 2000). Upregulation of ERAD components such as EDEM and derlin, which are induced by XBP1 in response to ER stress, increases the capacity for the removal of terminally unfolded/misfolded proteins thus relieving stress in the ER (Lee, Iwakoshi et al. 2003, Yoshida, Matsui et al. 2003, Oda, Okada et al. 2006). The concerted efforts of PERK, IRE1 and ATF6 are therefore designed to protect the cell from ER stress which may occur in cells during physiological processes such as protein folding in the endoplasmic reticulum.

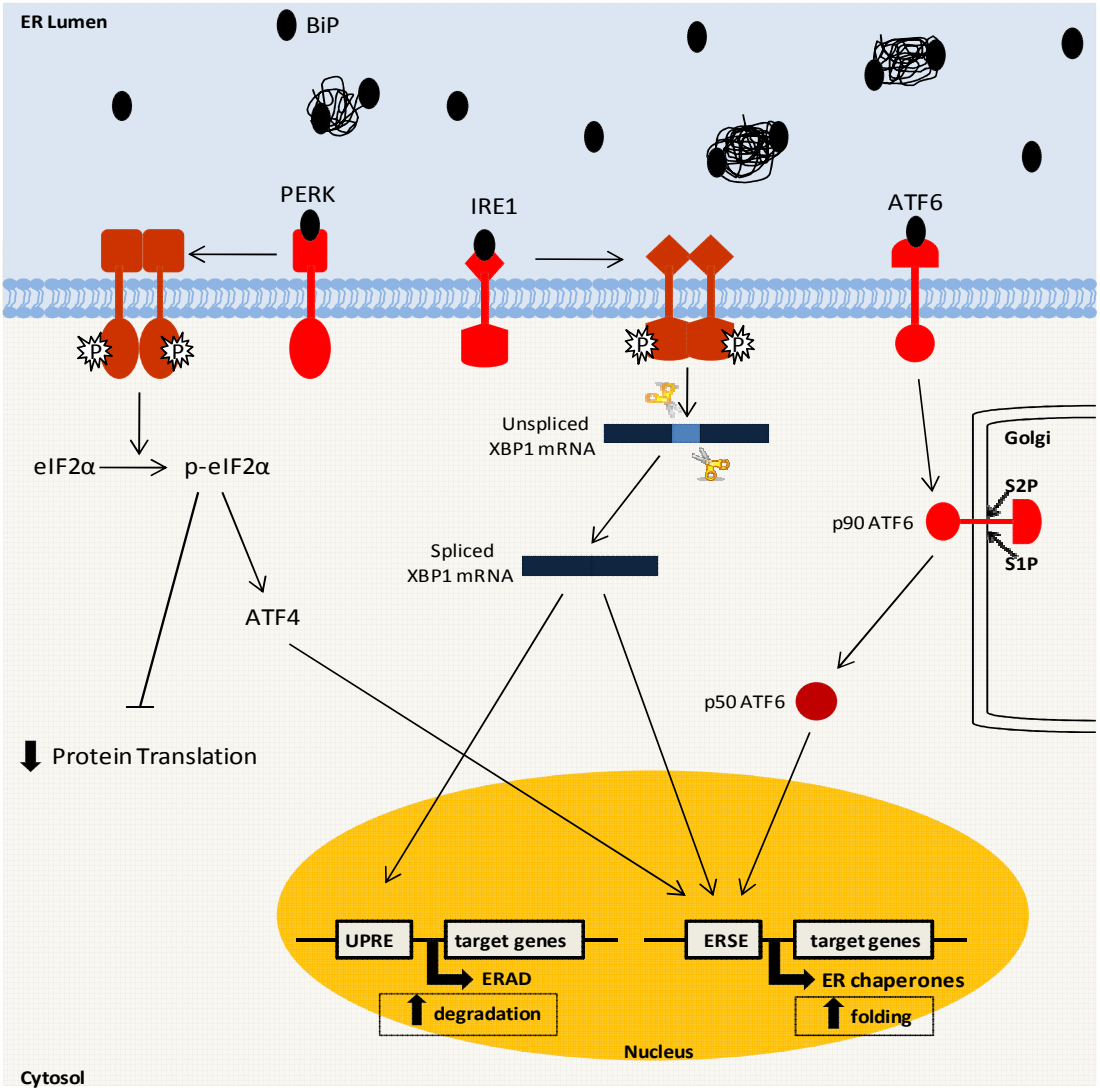


Figure 1.7 UPR signalling pathways in mammalian cells. The UPR is mediated by the activity of three ER resident transmembrane proteins (IRE1, PERK, and ATF6) which act in concert to regulate the UPR through their respective signalling cascades. All three proximal sensors are kept in an inactive conformation under basal conditions through their association with the ER resident chaperone protein, BiP. When unfolded proteins accumulate in the ER, BiP dissociates from the transducer proteins thereby permitting their activation. PERK is activated by dimerisation and phosphorylation, and phosphorylates eIF2α thereby inhibiting global protein translation. Phosphorylated eIF2α selectively promotes the translation of ATF4 which induces the expression of UPR target genes. IRE1 activation through dimerisation and phosphorylation results in the activation of its endoribonuclease activity and the splicing of XBP1 mRNA. Translation of spliced XBP1 mRNA results in the production of a transcription factor which upregulates target genes via the ERSE and UPR promoters. ATF6 activation involves translocation to the Golgi where it undergoes proteolytic cleavage by S1P and

S2P to generate an active 50-kDa transcription factor which upregulates the expression of target genes. Adapted from (Kitamura 2008).

1.5.4 The UPR in physiology

1.5.4.1 Adaptation of β-cell function to physiological demand

Dietary composition has changed dramatically in the last 25 years to favour energy-dense and saturated fat-enriched diets (Hill 2006). New diets coupled with commonly sedentary lifestyles of the 21st century have resulted in a disproportionate increase in the incidence of obesity and insulin resistance. As a result, the β-cell is constantly faced with the demand for nutrient-induced insulin synthesis which can be increased up to five fold in response to increased blood glucose levels (Goodge, Hutton 2000). To cope with varying demands for insulin synthesis under physiological conditions, the β-cell utilizes the UPR mechanism to balance the increase in protein synthesis with the protein folding capacity within the ER lumen. As a result of this, chaperone proteins such as BiP/GRP78, GRP94 and HYOU1 as well as the transducer proteins IRE1 and PERK are highly expressed in β-cells (Harding, Zeng et al. 2001, Kobayashi, Ogawa et al. 2000, Oyadomari, Takeda et al. 2001). Steady-state eIF2α phosphorylation in β-cells under physiological conditions exists as a result of the activation of PERK in response to an increase in protein load coupled with the subsequent dephosphorylation of eIF2α by the GADD34-PP1c complex (Novoa, Zeng et al. 2001, Brush, Weiser et al. 2003). An increase in glucose has been shown to acutely activate PERK and the subsequent phosphorylation of eIF2α (Gomez, Powell et al. 2008). The rapid (within 15 min) dephosphorylation of eIF2α stimulates the translation of insulin in response to glucose in β-cells (Vander Mierde, Scheuner et al. 2007). By this cycle of phosphorylation and dephosphorylation, PERK participates in the β-cell response to changes in blood glucose or nutrient levels under physiological conditions. IRE1α is also activated in β-cells in response to transient exposure to high glucose (Lipson K.L., Fonseca S.G et al. 2006). The phosphorylation and subsequent activation of IRE1α in response to glucose occurs independently of BiP dissociation and XBP1 splicing and requires the metabolism of glucose to stimulate insulin biosynthesis. The involvement of acute IRE1α activation in glucose stimulated insulin biosynthesis was demonstrated in a study in which the inhibition of IRE1α signalling by small interfering RNA resulted in the inhibition of insulin biosynthesis (Lipson K.L., Fonseca S.G et al. 2006). The acute

activation of PERK and IRE1 in the physiological response to increasing blood glucose and nutrient levels suggest that the early activation of some of the UPR components plays a role in the regulation of proinsulin and protein synthesis and in adapting the ER chaperone capacity to cope with the demand for protein synthesis.

1.5.4.2 Adaptation of β-cell mass to physiological demand

In addition to the activation of the UPR mechanism, the β-cell also attempts to compensate for increased metabolic load or obesity-associated insulin resistance by increasing its mass through hyperplasia and thus, an increase in its secretory capacity. Insulin resistance is defined by a state of decreased sensitivity to insulin and decreased efficiency of insulin signal transduction in peripheral tissues resulting in chronic hyperglycaemia. Elevated blood glucose levels signal the requirement for increased insulin secretion and synthesis from the β-cell and have been suggested to modulate the increase in β-cell mass. Indeed, *in vivo* experiments have shown that glucose infusion in rats and mice resulted in an increase in β-cell mass (Bernard, Berthault et al. 1999; Paris, Bernard-Kargar et al. 2003; Alonso, Yokoe et al. 2007). Glucose mediated cell proliferation has also been reported in a study in INS-1 cells (Hügl, White et al. 1998). The signalling pathways through which glucose induces an increase in β-cell mass are not entirely clear. Several mechanisms have been proposed including the activation of the insulin receptor and the downstream activation of PKB/Akt signalling which serves a role in β-cell proliferation (Bernal-Mizrachi, Wen et al. 2001, Tuttle, Gill et al. 2001). The effect of insulin receptor signalling on β-cell mass has been assessed in separate studies. Insulin infusion in mice was shown to induce β-cell proliferation and an increase in β-cell mass (Paris, Bernard-Kargar et al. 2003). MIN6 cells with an 80% knockdown of the insulin receptor were also reported to show reduced proliferation (Ohsugi, Cras-Méneur et al. 2005). These studies suggest that glucose induced insulin stimulates β-cell proliferation to cope with physiological demand. Glucose has also been suggested to induce the activation of the mTOR signalling pathway by increasing the intracellular levels of ATP and the subsequent inactivation of AMPK. The mTOR signalling pathway has been shown to be an important regulator

of β-cell mass and proliferation (Heit, Apelqvist et al. 2006, Jhala, Canettieri et al. 2003).

1.6 ER stress and apoptosis

Despite the adaptive signals transmitted by the proximal sensors under ER stress conditions, the activation of the UPR may sometimes prove insufficient to restore homeostatic balance within the ER (Eizirik, Cardozo et al. 2008). Chronic unresolved ER stress results in the progressive accumulation of unfolded/misfolded proteins in the ER lumen and consequently, gross disruption of ER homeostasis which culminates in cell death. Cell death occurs through an organised process known as apoptosis. Apoptosis is a process of programmed cellular suicide composed of a complex of synchronised factors which interact in a tightly controlled and genetically regulated manner (Groenendyk, Michalak 2005). Apoptosis is employed by healthy organisms to remove damaged or unwanted cells and thus plays an important role in tissue homeostasis, development and defence against pathogens (Danial, Korsmeyer 2004, Meier, Finch et al. 2000, Siegel, Muppidi et al. 2003). Apoptosis also plays an important role in the pathogenesis of human diseases with its inhibition resulting in disease states such as cancers and autoimmune disorders (Green, Evan 2002). The augmentation of apoptosis could also result in the development of diseases such as Alzheimer's, Parkinson's and diabetes (Mattson 2000).

1.6.1 Apoptotic signalling

Apoptosis is stimulated by intrinsic or extrinsic signals as well as pathological cellular signals, all of which culminate in the activation of cysteine proteases known as caspases (Siegel, Muppidi et al. 2003, Strasser, O'Connor et al. 2000). Caspases are cysteine-dependent aspartate-specific proteases which contain a highly conserved pentapeptide active site (QACRG) (Groenendyk, Michalak 2005). Caspases are split into two functional groups: the initiator caspases made up of caspase-2, -8, -9, -10 and -12 and effector/executioner caspases made up of caspase-3, -6 and -7 (Nicholson, Thornberry 1997, Nakagawa, Yuan 2000, Denault, Salvesen 2002). As a result of their role in apoptotic signalling, caspases are initially produced in the cell as inactive

zymogens or pro-enzymes which require cleavage for their activation. The activation of a caspase cascade involves the initial cleavage of an initiator caspase followed by subsequent activation of several caspases and culminating in the cleavage and activation of an effector caspase. Activated effector caspases induce apoptosis by acting directly on cellular components such as the cytoskeleton (Kluck, Bossy-Wetzel et al. 1997), and ion transporters (Remillard, Yuan 2004) to induce apoptosis (Slee, Adrain et al. 1999). The importance of caspases in cell survival has been studied in mouse models with a deficiency in caspase-3 and caspase-9 resulting in embryonic lethality as a result of insufficient brain development (Kuida, Zheng et al. 1996, Kuida, Haydar et al. 1998). Caspase activation is mediated by signals which emanate from both the extrinsic and intrinsic apoptotic pathways.

1.6.1.1 The extrinsic apoptotic pathway

In the extrinsic apoptotic signalling pathway, death receptors such as the tumor necrosis factor receptor (TNFR) and the Fas receptor (FasR) recruit an initiator caspase such as caspase-8 to their cytoplasmic domain (Ashkenazi 2002). On ligand binding to the death receptor, an adaptor protein such as tumor necrosis factor receptor associated death domain (TRADD) or Fas associated death domain (FADD) protein is recruited resulting in the proximity induced dimerisation and activation of the initiator caspase (Donepudi, Sweeney et al. 2003, Boatright, Renatus et al. 2003). The activation of an initiator caspase results in the activation of a caspase cleavage and activation cascade which culminates in the activation of an effector caspase to induce apoptotic signals.

1.6.1.2 The intrinsic apoptotic pathway

An intrinsic stimulus of apoptosis is induced by pathological cellular signals such as DNA damage. This pathway involves the mitochondria, caspases and the BCL-2 family of proteins. The BCL-2 family of proteins is made up of both pro- and anti-apoptotic members. The anti-apoptotic members include BCL-2, BCL-$_{XL}$, BCL-w and MCL-1 (Schroder 2008). The pro-apoptotic family members are subdivided into three classes, the multidomain members such as BAX, BAK and BCL-xs, single BCL-2 homology 3

(BH3) domain-only proteins such as BIM, BAD, BID, PUMA and NOXA (Karst, Li 2007, Häcker, Weber 2007) and proteins with a less well conserved BH3 domain called BNIPs (Zhang, Cheung et al. 2003). Anti-apoptotic BCL-2 proteins are bound to the pro-apoptotic BID and BIM under basal conditions to inhibit their activity (Morishima, Nakanishi et al. 2004). In the direct binding model, apoptotic stimuli induce the transcriptional induction, post-translational modification and decreased synthesis of anti-apoptotic BCL-2 family members (Karst, Li 2007, Häcker, Weber 2007), resulting in the release of BIM and BID from their inhibitory interaction with the anti-apoptotic family members. Released BIM and BID, bind to and directly activate BAK and BAX thereby stimulating the induction of apoptosis (Häcker, Weber 2007). In the displacement model, BAK and BAX are sequestered at the mitochondria by the anti-apoptotic MCL1 and BCL-$_{XL}$. In response to apoptotic stimuli, activated BH-3 only proteins displace BAK and BAX from MCL1 and BCL-$_{XL}$, thereby stimulating their activation (Karst, Li 2007, Häcker, Weber 2007). Once activated, BAK and BAX promote apoptosis through oligomerisation and subsequent insertion into the mitochondrial membrane. This results in the formation of a pore through which cytochrome *c* is released from the mitochondria into the cytosol (Häcker, Weber 2007). In the cytosol, cytochrome *c* forms a complex known as an apoptosome by interacting with APAF1 and procaspase-9. Formation of the apoptosome induces the cleavage and activation of caspase-9 which initiates a proteolytic caspase cascade and the eventual activation of the effector caspase, caspase-3. In addition to inducing the release of cytochrome *c* from the mitochondria, BAK and BAX also induce the release of Ca^{2+} from the ER by oligomerisation and insertion at the ER membrane (Zong, Li et al. 2003, Scorrano, Oakes et al. 2003). Ca^{2+} release from the ER may also be induced by the binding of cytochrome *c* to the IP_3R on the ER membrane (Groenendyk, Michalak 2005). An increase in cytosolic Ca^{2+} triggers further release of cytochrome *c* from the mitochondria (Crompton 1999). Additionally, an increase in cytosolic Ca^{2+} levels results in the augmentation of calpain activity (Nakagawa, Yuan 2000). Calpains are cytoplasmic Ca^{2+}-dependent cysteine proteases which are ubiquitously expressed in all animals (Groenendyk, Michalak 2005). Calpains are active under basal conditions and function in cellular processes such as cell cycle (Choi, Lee et al. 1997) and cellular

remodelling (Potter, Tirnauer et al. 1998). Under conditions of increased cytosolic Ca^{2+}, calpain cleaves the initiator procaspase-12 resulting in the generation of active caspase-12 (Nakagawa, Yuan 2000). Activated caspase-12 is capable of the direct cleavage of caspase-9 in the cytosol independent of the mitochondrial cytochrome *c*/APAF1 pathway. The active caspase-9 in turn, cleaves and activates the effector caspase, caspase-3 (Morishima, Nakanishi et al. 2002, Tan, Dourdin et al. 2006). While the role of caspase-12 in the context of ER stress induced apoptosis is fully characterised in mice, it is reported to be redundant in humans (Szegezdi, Fitzgerald et al. 2003, Lamkanfi, Festjens et al. 2006). In its place, it is suggested that caspase-4 is involved in ER stress induced apoptosis in human cells (Hitomi, Katayama et al. 2004).

1.6.2 Chronic ER stress induced apoptosis

Chronic ER stress can lead to the induction of apoptosis by several pathways which involve the UPR transducer proteins. While the exact mechanisms are unclear, it has been established that the chronic induction of ER stress signalling results in the switch from protective to apoptotic signals mediated by the same transducer proteins.

1.6.2.1 CHOP mediated apoptotic signalling

The expression of CHOP downstream of the PERK pathway has been implicated in the induction of apoptosis in chronically ER stressed cells. CHOP, also known as GADD153/DDIT3, is a bZIP transcription factor of the C/EBP family (Ron, Habener 1992), which is directly upregulated downstream of ATF4 (Harding, Novoa et al. 2000b). ATF4 undergoes heterodimerisaton with a CCAAT/enhancer binding protein-β (C/EBPβ) to stimulate the expression of CHOP under ER stress conditions (Fawcett, Martindale et al. 1999). While its expression level is low under basal conditions, CHOP is significantly upregulated in response to ER stress (Oyadomari, Mori 2003) and other cellular stresses such as nuclear DNA damage (Eizirik, Björklund et al. 1993). The function of CHOP in the propagation of apoptotic signals was demonstrated by its deletion in mice which resulted in a reduction in ER stress induced apoptosis (Zinszner, Kuroda et al. 1998). Overexpression of CHOP has also been reported to be sufficient for the induction of apoptosis in several cell lines (McCullough, Martindale et al. 2001).

As it is a transcription factor, CHOP cannot induce apoptosis directly. It therefore functions to sensitise cells to apoptosis via several mechanisms. CHOP has been suggested to augment apoptosis in ER stressed cells by upregulating the expression of ERO-1, a thiol oxidase which re-oxidises PDIs to promote protein folding in the ER (Marciniak, Yun et al. 2004). While the protein folding function of ERO-1 is protective to the β-cell, the generation of ROS as a by-product may contribute to the stimulation of apoptosis by inducing oxidative stress (Marciniak, Yun et al. 2004). Chronic ER stress has been shown to perturb the balance between pro-and anti-apoptotic BCL-2 family proteins with pro-apoptotic BH3-only proteins such as BIM (Puthalakath, O'Reilly et al. 2007), PUMA and NOXA (Reimertz, Kögel et al. 2003, Li, Lee et al. 2006) transcriptionally upregulated under ER stress conditions. CHOP mediated transcriptional upregulation of BIM has been implicated in the induction of apoptosis (Puthalakath, O'Reilly et al. 2007) in cells treated with ER stress inducers such as thapsigargin and tunicamycin which induce ER stress by inhibiting the SERCA pumps on the ER and inhibiting N-linked glycosylation respectively (Lytton, Westlin et al. 1991). CHOP mediated BIM upregulation induces the activation of BAK and BAX and the downstream activation of the caspase cascade through the formation of an apoptosome and caspase-9 activation. CHOP induced BIM expression therefore serves as an indirect mechanism for caspase activation in chronically ER stressed cells. CHOP has also been shown to mediate the downregulation of anti-apoptotic BCL-2 in ER stressed cells thereby augmenting apoptosis (McCullough, Martindale et al. 2001) and perturbing cellular redox by inducing the depletion of cellular glutathione as BCL-2 is involved in the regulation of glutathione metabolism (McCullough, Martindale et al. 2001). Other mechanisms through which CHOP may induce apoptosis under conditions of chronic ER stress include the upregulation of the death receptor 5 (Yamaguchi, Wang 2004) and the induction of TRB3 (tribbles 3), a protein kinase B inhibitor which has been shown to contribute to ER stress induced apoptosis (Ohoka, Yoshii et al. 2005). Thus, ER stress induced apoptosis signalling through the PERK pathway is transmitted both by mitochondria dependent and independent pathways.

1.6.2.2 IRE1 mediated apoptotic signalling

Chronic ER stress mediated apoptosis is also transmitted by signals emanating from activated IRE1. Chronic activation of IRE1 has been reported to result in the recruitment of the adaptor protein TNF receptor-associated factor 2 (TRAF2) to the cytosolic domain of IRE1 (Urano, Wang et al. 2000). The interaction of TRAF2 with IRE1 allows for apoptotic signalling via two separate mechanisms. Firstly, the association of TRAF2 with IRE1 results in the clustering of procaspase-12 at the ER membrane as TRAF2 exists in a latent but stable cytosolic complex with procaspase-12 under basal conditions (Yoneda, Imaizumi et al. 2001). Clustering of procaspase-12 results in proximity induced dimerisation/oligomerisation of the prodomains and the subsequent activation of the caspase activity (Yoneda, Imaizumi et al. 2001). The association of IRE1 with TRAF2 also results in the recruitment of a third component known as apoptosis signal-regulating kinase 1 (ASK1) to the complex (Nishitoh, Saitoh et al. 1998). The importance of ASK1 in this complex is demonstrated in a study in which neurons from $ASK^{-/-}$ mice were found to be resistant to ER stress induced cell death (Nishitoh, Matsuzawa et al. 2002). The IRE1α-TRAF2-ASK1 complex induces the phosphorylation of the c-Jun N-terminal kinase (JNK) thereby activating the kinase (Nishitoh, Saitoh et al. 1998, Nishitoh, Matsuzawa et al. 2002). Activated JNK induces apoptosis by phosphorylating the pro-apoptotic BH3-only BCL2 family member, BIM thereby releasing BIM from its inhibitory interactions and protecting against proteasomal degradation (Morishima, Nakanishi et al. 2004). Released BIM associates with pro-apoptotic BAK and BAX thereby stimulating their activity and the subsequent activation of the caspase cascade (Lei, Davis 2003, Putcha, Le et al. 2003). Apoptotic signalling is also enhanced in this complex by ASK1 induced phosphorylation of the anti-apoptotic BCL2 protein resulting in its deactivation (Yamamoto, Ichijo et al. 1999).

While signalling pathways downstream of PERK and IRE1 coupled with ER Ca^{2+} levels have been reported to be involved in ER stress induced apoptosis, the precise mechanisms through which this process occurs is unknown. This is because many of the proposed mechanisms for the induction of apoptosis are cell and context dependent and as such do not represent the full article. While apoptosis is induced

under chronic ER stress conditions, cells exposed to mild or acute ER stress may adapt and survive (Rutkowski, Arnold et al. 2006). A study into ER stress induced apoptosis in a cell culture system suggested that the decision between cell death and adaptation to ER stress is dependent on the intensity of the activation of the UPR sensors rather than the specific pathways activated in response to ER stress (Rutkowski, Arnold et al. 2006). Rutkowski et al. showed that both pro- and anti-apoptotic factors were induced under conditions of mild and chronic ER stress. Mild ER stress however favoured prosurvival and adaptation of the cells as a result of the low stability of pro-apoptotic mRNAs and proteins in comparison to pro-survival and adaptation factors (Rutkowski, Arnold et al. 2006). Under conditions of chronic ER stress however, pro-apoptotic factors are maintained at high levels resulting in the activation of apoptosis signalling pathways. Chronic physiological adaptation of the β-cell to increased metabolic load, which may occur under conditions of obesity or insulin resistance, may also lead to the induction of apoptosis. Chronic (12-24 h) eIF2α phosphorylation in β-cells in response to an increase in protein load has been reported to lead to β-cell dysfunction and apoptosis (Cnop, Ladriere et al. 2007). Chronic (3-7 days) high glucose exposure has also been shown to induce IRE1 activation coupled with XBP1 splicing and the degradation of insulin mRNA (Lipson, et al. 2006). Apoptotic signalling and the degradation of nascent insulin mRNA by chronically activated IRE1 in response to high blood glucose and nutrient levels may be one of the mechanisms through which β-cells are lost in type 2 diabetes and obesity.

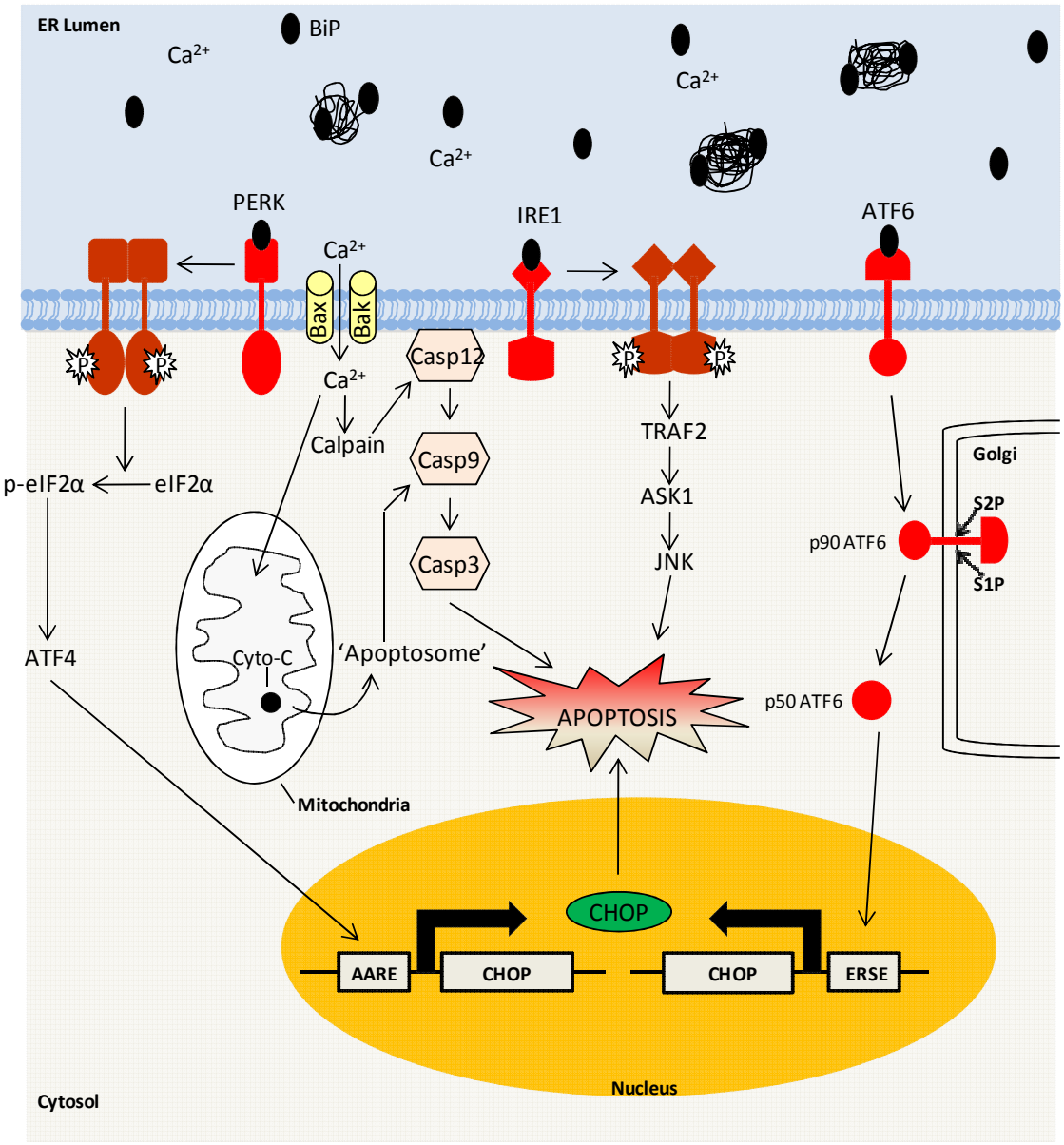


Figure 1.8 ER stress pathways implicated in the induction of cell apoptosis. Activation of PERK-eIF2α pathway induces ATF4 expression. ATF4 activates the amino acid response element (AARE) thereby inducing CHOP expression. The ATF6 (and the IRE1) pathway may also induce the expression of CHOP through the activation of the ER stress response element (ERSE). CHOP activation leads to the upregulation of pro-apoptotic and downregulation of anti-apoptotic Bcl-2 family members. ER stress induces the oligomerisation of pro-apoptotic Bak and Bax at the ER membrane, resulting in the release of Ca^{2+} from the ER. An increase in cytosolic Ca^{2+} results in the activation of calpain and the subsequent cleavage of procaspase-12 to caspase-12. Ca^{2+} may also stimulate the release of mitochondrial cytochrome *c* resulting in the formation of an apoptosome and the cleavage and activation of procaspases-9 to caspase-9. Activated IRE1 interacts with TRAF2, allowing for the recruitment and activation of ASK-1 and downstream JNK phosphorylation which results in apoptotic signalling. Adapted from (Kitamura 2008).

1.7 ER stress and disease

The accumulation of misfolded proteins and protein aggregates in the cytoplasm and the progressive loss of specific types of neurons have been observed in disease states such as Parkinson's, Alzheimer's and Huntington's diseases (Forman, Lee et al. 2003, Lindholm, Wootz et al. 2006). In these disease states, protein accumulation in the ER and activation of the UPR were also observed indicating a role for chronic ER stress induced apoptosis in the disease pathology. Chronic ER stress has also been implicated in the development of type III autosomal dominant retinitis pigmentosa (ADRP) (Humphries, Kenna et al. 1992, Colley, Cassill et al. 1995). The accumulation of misfolded rhodopsin, which occurs as a result of mutations in the protein, results in chronic ER stress induction and the activation of apoptosis leading to retinal degeneration in both humans and drosophila (Colley, Cassill et al. 1995, Davidson, Steller 1998). Chronic ER stress induced apoptosis has also been implicated in the gross loss of β-cells in obesity associated type 2 diabetes (Harding, Zeng et al. 2001, Delepine, Nicolino et al. 2000).

1.7.1 Diabetes mellitus

Diabetes Mellitus is a complex disease characterised by a reduction in pancreatic β-cell mass due to an increase in β-cell apoptosis and defective β-cell regeneration. The loss of functional β-cell mass culminates into the development of hyperglycaemia. Diabetes is a major cause of morbidity and mortality, decreasing both life expectancy and quality in affected individuals. The two main forms of diabetes are type 1 diabetes (T1D) and type 2 diabetes (T2D). Type 1 diabetes is characterised by a complete lack of insulin secretion due to the progressive destruction of the pancreatic β-cells (Gillespie 2006). T1DM occurs as a result of an autoimmune-mediated process where a chronic inflammation known as insulitis, induces the destruction of β-cells (Eizirik, Mandrup-Poulsen 2001). Immune cells produced as a result of inflammation release cytokines and other factors which trigger secondary pathways of cell death in the β-cells (Gillespie 2006, Mathis, Vence et al. 2001).

1.7.2 Type 2 diabetes and obesity

At least 150 million people are affected with T2DM worldwide, a figure projected to double by 2025 (Zimmet, Alberti et al. 2001). T2DM usually presents in the middle-aged and elderly but is now increasingly diagnosed in children and young adults. Type 2 diabetes is characterised by a progressive decline in β-cell secretory function and chronic insulin resistance in peripheral tissues. Obesity is almost invariably linked with insulin resistance and is therefore considered a major risk factor in the development of T2DM (Ludvik, Nolan et al. 1995, Burke, Williams et al. 1999). Indeed, research figures have shown that approximately 80% of diabetics are obese (Bloomgarden 2000). As a result of obesity, elevated levels of circulating FFAs in the blood have been implicated in the development of insulin resistance which is suggested to precede the development of hyperglycaemia (Martin, Warram et al. 1992). However, a subset of people who are obese and relatively insulin resistant do not develop diabetes but instead, compensate by increasing insulin secretion from the β-cells (Polonsky 2000). The adaptive increase in β-cell secretory capacity mediated by an increase in β-cell mass has been reported in rodent models of obesity without diabetes (Flier, Kulkarni et al. 2001). An adaptive increase in β-cell mass was also reported in non-diabetic obese human subjects (Ogilvie 1933, Klöppel, Löhr et al. 1985). Type 2 diabetes may develop in genetically predisposed individuals who are unable to sustain the β-cell compensatory response (Prentki & Nolan, 2006). In this group of people, the initial compensation for insulin secretion is followed by progressive deterioration in β-cell function (Leahy 2005), associated with the loss of β-cell mass through apoptosis (Butler, Janson et al. 2003a). Several mechanism(s) through which apoptosis is induced in the β-cells of obesity associated type 2 diabetics have been proposed.

1.7.3 Mechanisms of FFA induced β-cell dysfunction and apoptosis in type 2 diabetes and obesity

Data collated from autopsies suggest that the progressive decline in insulin secretion in type 2 diabetes is accompanied by a simultaneous decrease in β-cell mass which occurs as a result of an increase in β-cell apoptosis (Butler, Janson et al. 2003a). High levels of circulating FFAs have been implicated in the development of β-cell

dysfunction and induction of apoptosis in human subjects and animal models of obesity and diabetes (Shimabukuro, Zhou et al. 1998, Boden, Shulman 2002, Kashyap, Belfort et al. 2003). Although FFAs acutely stimulate insulin secretion, prolonged exposure of β-cells to high FFA levels reduces their responsiveness both *in vitro* (Zhou, Grill 1994, Zhou, Grill 1995, Zhou, Grill 1995, Bollheimer, Skelly et al. 1998) and *in vivo* (Sako, Grill 1990, Carpentier, Mittelman et al. 1999, Paolisso, Gambardella et al. 1995). In addition to inducing β-cell dysfunction, chronic exposure to FFAs has also been reported to induce β-cell apoptosis (Kharroubi, Ladriere et al. 2004, Cunha, Hekerman et al. 2008, Laybutt, Preston et al. 2007). Saturated long chain fatty acids such as palmitate and to a lesser extent, unsaturated long chain fatty acids such as oleate have been reported to induce apoptosis in clonal β-cell lines (Kharroubi, Ladriere et al. 2004, Cunha, Hekerman et al. 2008), primary rat β-cells (Cnop, Hannaert et al. 2001, Cnop, Hannaert et al. 2002, Maedler, Spinas et al. 2001) and human islets (Cunha, Hekerman et al. 2008, Ladrière, Igoillo-Esteve et al. 2010). FFAs may therefore contribute to the development of β-cell dysfunction and the subsequent apoptosis of the β-cell. While the mechanisms through which apoptosis is induced are currently unclear, several mechanisms have been proposed. These include ceramide synthesis, chronically elevated Ca^{2+} levels, amyloid formation, oxidative stress and ER stress.

1.7.3.1 Ceramide synthesis

Saturated FAs such as palmitate may induce β-cell apoptosis through the *de novo* synthesis of ceramide in the ER (Shimabukuro, Higa et al. 1998). Ceramide biosynthesis is catalysed by serine palmitoyl transferase (SPT), an enzyme which induces the condensation of palmitate and serine to form 3-dehydrosphinganine, a precursor of sphingosine (Weiss, Stoffel 1997). Sphingosine undergoes acetylation to generate a molecule of ceramide which contains two molecules of long chain fatty acids. Although the exact mechanisms through which ceramide induces apoptosis are unclear, it has been suggested that it induces the activation of JNK, thus promoting apoptotic signalling. Ceramide has also been proposed to stimulate apoptosis through the inhibition of mitochondrial respiratory chain complexes I and III thereby inducing oxidative stress (Schönfeld, Wojtczak 2007, Gudz, Tserng et al. 1997). Evidence for the

involvement of ceramide in FFA induced apoptosis was published in a study in which the inhibition of SPT activity resulted in the inhibition of ceramide synthesis and partial protection from apoptosis in β-cells isolated from Zucker Diabetic Fatty (ZDF) rats (Shimabukuro, Zhou et al. 1998, Shimabukuro, Higa et al. 1998). The treatment of islet cells with ceramide analogues has also been reported to result in the loss of cell viability (Maedler, Spinas et al. 2001).

1.7.3.2 Chronically elevated intracellular calcium

Chronic hyperglycaemia has been shown to lead to long term increases in cytosolic Ca^{2+} levels which can induce β-cell dysfunction and apoptosis. Exposure of human islets to high glucose for 48 h resulted in the persistent elevation of cytoplasmic Ca^{2+} which was partially restored by diazoxide, an opener of K^+ ion channels which results in the inhibition of Ca^{2+} ion channels (Björklund, Lansner et al. 2000). FFAs have also been shown to mediate the depletion of ER Ca^{2+} levels and a concomitant rise in cytosolic Ca^{2+} levels (Gwiazda, Yang et al. 2009). High levels of intracellular Ca^{2+} may induce apoptosis through several mechanisms. Intracellular Ca^{2+} is capable of dissipating into the nucleus through the nuclear pore thereby equilibrating with the levels in the cytosol. In the nuclei, Ca^{2+} modulates the transcription of genes (Gong, Blok et al. 1995) and nucleases (Ermak, Davies 2002) involved in cellular apoptosis thereby stimulating apoptotic signalling. An increase in intracellular Ca^{2+} levels could also result in the activation of calpains, the Ca^{2+} dependent cysteine protease which functions to cleave procaspase-12 thereby activating the caspase cascade (Nakagawa, Yuan 2000). Increasing intracellular Ca^{2+} could also be taken up by the mitochondria resulting in Ca^{2+} accumulation within the mitochondria and the consequential release of cytochrome *c* from the mitochondria allowing for apoptosome formation and activation of caspase-9.

1.7.3.3 Islet amyloid formation

The loss of β-cells in type 2 diabetes has also been attributed to the appearance of islet amyloid (Clark, Wells et al. 1988, Johnson, O'Brien et al. 1989) derived from islet amyloid polypeptide (IAPP) (Cooper, Willis et al. 1987, Westermark, Wernstedt et al.

1987). IAPP is a 37-amino acid peptide which is co-expressed and co-secreted with insulin by pancreatic β-cells (Butler, Chou et al. 1990). Apart from amino acids 20-29, IAPP is highly conserved between species. The 20-29 peptide region is homologous in humans, cats and monkeys and confers amyloidogenic properties on the protein (Westermark, Engström et al. 1990, Lorenzo, Razzaboni et al. 1994). In contrast, this region has proline substitutions in rats and mice and as such, murine IAPP is soluble and non-amyloidogenic (Lorenzo, Razzaboni et al. 1994). Fibrillar islet amyloid deposits are characteristic features of the islets in type 2 diabetes (Ritzel, Butler 2003) and have been implicated in β-cell death. Although mice do not spontaneously develop type 2 diabetes, the expression of human IAPP is both mice and rats resulted in the development of diabetes as a result of β-cell loss through an increase in β-cell apoptosis (Butler, Janson et al. 2003b, Butler, Jang et al. 2004). IAPP-induced apoptosis was also detected in INS-1 cells and human IAPP (HIP) transgenic rats (Huang, Lin et al. 2007). More significantly, large oligomers, adjacent to amyloid oligomers, were detected in pancreatic tissue obtained from human diabetic subjects in comparison to non-diabetic subjects correlating with the expression of cleaved caspase-3, a marker of apoptosis (Zhao, Sui et al. 2009). Although the exact mechanism(s) through which islet amyloid deposits mediate cell death is unknown, it is apparent that the toxic oligomers that induce β-cell apoptosis are small IAPP oligomers which precede the development of IAPP fibrils and function as non-selective ion channels (Mirzabekov, Lin et al. 1996, Janson, Ashley et al. 1999). Although islet amyloid has been implicated in β-cell death, it is unclear to what extent this process induces β-cell apoptosis in the development of type 2 diabetes.

1.7.3.4 Oxidative stress

FFA induced β-cell apoptosis may also be stimulated by the generation of ROS as a result of fatty acid metabolism (Piro, Anello et al. 2002, Kaneto, Katakami et al. 2007). High levels of circulating FAs, which may occur in type 2 diabetes and obesity, may result in increased FA metabolism through mitochondrial oxidation. Increased FA oxidation stimulates an increase in mitochondrial membrane potential and superoxide production in the cell (Koshkin, Dai et al. 2008). The β-cell does not possess a strong

defence mechanism against the action of ROS as expression levels of ROS-detoxifying enzymes are low in the β-cell (Grankvist, Marklund et al. 1981, Robertson, Harmon et al. 2004, Sigfrid, Cunningham et al. 2004). Elevated ROS in the cell may therefore induce oxidative stress which is capable of inhibiting insulin signalling through the activation of the protein kinase JNK (Kaneto, Katakami et al. 2007). JNK phosphorylation of IRS-1 on serine 307 reduces insulin receptor stimulated tyrosine phosphorylation and subsequently, insulin signalling (Kaneto, Katakami et al. 2007). Oxidative stress also leads to a decrease in the transcription of the insulin gene by decreasing Pdx1 and Maf A, both transcription factors necessary for the transcription of insulin (Olson, Redmon et al. 1993). In addition to inducing β-cell dysfunction, JNK phosphorylation also induces the activation of apoptotic pathways, indicating a role for FFA induced oxidative stress in the loss of β-cells in T2DM and obesity (Morishima, Nakanishi et al. 2004). While some studies provide evidence that FFA-mediated ROS generation may be involved in β-cell apoptosis, a general consensus has not been reached. A study by Cnop et al., suggests that FFA cytotoxicity was not dependent on mitochondrial FFA oxidation as the inhibition of CPT1 did not alter FFA induced β-cell apoptosis (Cnop, Welsh et al. 2005). In the same study, the application of antioxidant agents or free radical scavenging compounds did not inhibit FFA induced β-cell apoptosis suggesting that oxidative stress does not play a role in FFA mediated β-cell apoptosis. Significant levels of ROS have however been reported in the islets of type 2 diabetics in comparison to controls, and the levels of these markers were shown to correlate with the degree of impairment in insulin secretion in response to glucose (Lupi, Dotta et al. 2002, D'Aleo, Del Guerra et al. 2009). Additional evidence for glucose stimulated ROS generation was published in the same study showing a decrease in ROS levels and islet dysfunction in response to the overexpression of antioxidant enzymes in the presence of glucose. A similar improvement in β-cell function was reported in a separate study in which isolated islets from diabetic human subjects were treated with antioxidant agents (Del Guerra, Lupi et al. 2005). While the role of FFA induced oxidative stress in the loss of β-cells is unclear, these studies suggest a role for oxidative stress in the loss of β-cells in T2DM and obesity.

1.7.3.5 ER stress

In vitro, FFAs have been shown to induce ER stress and as a consequence, the UPR in islets and β-cell lines (Kharroubi, Ladriere et al. 2004, Cunha, Hekerman et al. 2008). Circulating FFAs in the blood are comprised of a mixture of unsaturated and saturated fatty acids of which the unsaturated fatty acid oleate, and the saturated fatty acid, palmitate are predominant (Richieri, Kleinfeld 1995). Several studies have shown that saturated and unsaturated fatty acids elicit quantitatively and qualitatively different effects on ER stress signalling (Cunha, Hekerman et al. 2008, Laybutt, Preston et al. 2007, Diakogiannaki, Welters et al. 2008). Whilst oleate and palmitate are predominantly used in ER stress studies *in vitro*, other saturated and unsaturated long chain fatty acids exhibit the same profile of differential ER stress induction (Diakogiannaki, Welters et al. 2008, Welters, Tadayyon et al. 2004, Diakogiannaki, Dhayal et al. 2007). The saturated fatty acid palmitate has been shown to be more potent than oleate in the induction of ER stress in clonal β-cell lines, primary rodent β-cells and human islets (Cunha, Hekerman et al. 2008, Ladrière, Igoillo-Esteve et al. 2010). Time course studies in MIN6 and INS-1E cells showed that palmitate induced PERK and eIF2α phosphorylation, inhibition of protein synthesis and the induction of CHOP expression (Cunha, Hekerman et al. 2008, Laybutt, Preston et al. 2007). These ER stress responses were not significant in cells treated with oleate (Laybutt, Preston et al. 2007) or a non-toxic equimolar mixture of oleate and palmitate (Cunha, Hekerman et al. 2008). The IRE1α pathway is also preferentially activated by palmitate as opposed to oleate as evidenced by XBP1 splicing (Cunha, Hekerman et al. 2008, Laybutt, Preston et al. 2007) Palmitate treatment has also been reported to induce the expression of chaperone proteins such as BiP/GRP78, GRP94, HYOU1 and Dnajb9 in MIN6 cells (Laybutt, Preston et al. 2007), although one study reported that BiP was not induced in response to palmitate exposure in INS-1 cells (Karaskov, Scott et al. 2006). In contrast, the activation of ATF6 and the induction of chaperone protein expression has been reported in response to both oleate and palmitate treatment in INS-1E cells (Cunha, Hekerman et al. 2008).

While the exact mechanism(s) through which FFAs induce ER stress is unknown, several processes may be involved. FFAs have been proposed to induce ER stress in β-cells through changes in the ER Ca^{2+} homeostasis (Cunha, Hekerman et al. 2008; Gwiazda, Yang et al. 2009). Palmitate induces the depletion of Ca^{2+} from the ER through the inhibition of the SERCA pump in a mechanism which resembles the action of thapsigargin (Lytton, Westlin et al. 1991; Gwiazda, Yang et al. 2009). The depletion of ER Ca^{2+} results in the disruption of ER homeostasis and may impair protein folding processes resulting in the accumulation of misfolded/unfolded proteins in the ER lumen (Cnop et al. 2010). Palmitate is also proposed to induce ER stress through the inhibition of ER-to-Golgi trafficking of newly synthesised proteins (Preston, Gurisik et al. 2009). Inhibition of protein trafficking causes the build up of proteins in the ER and as a result, the induction of ER stress (Preston, Gurisik et al. 2009). Both the depletion of ER Ca^{2+} and the inhibition of ER-to-Golgi protein trafficking may be stimulated by changes in the triglyceride composition of the ER membrane following exposure to FFAs (Cnop et al. 2010). Palmitate has also been reported to induce the rapid degradation of carboxypeptidase E (CPE), an enzyme involved in the processing of newly synthesised proinsulin (Jeffrey, Alejandro et al. 2008). CPE degradation may result in the accumulation of unprocessed proinsulin in the secretory pathway and thus induce ER stress (Jeffrey, Alejandro et al. 2008).

The induction of chronic ER stress in response to elevated FFA levels is also proposed to be a mechanism through which β-cell death is induced in type 2 diabetes and obesity. Evidence for the role of ER stress in palmitate induced β-cell apoptosis was presented in a study in which BiP/GRP78 overexpression in MIN6 cells induced a milder ER stress response and partial protection against palmitate induced β-cell apoptosis (Laybutt, Preston et al. 2007). Apoptosis in response to FFA exposure in β-cell lines may be mediated by an increase in the expression of CHOP, a downstream of target of PERK activation, JNK activation, a downstream target of IRE1 and/or the cleavage and subsequent activation of caspase-3 (Laybutt, Preston et al. 2007). Following the suggestion that FFA induced ER stress plays a role in the induction of β-cell apoptosis, some studies have attempted to protect β-cells by altering the course of the UPR in response to FFA exposure. In one study, a deficiency in PERK to eIF2α

signalling due to a homozygous mutation at the eIF2α phosphorylation site (Ser51Ala) in mice was reported to be detrimental to β-cell survival (Scheuner, Song et al. 2001). In another study, tissue specific PERK knockout in mice also resulted in defects in β-cell proliferation resulting in low β-cell mass (Zhang, Feng et al. 2006). In contrast, other studies employed salubrinal, a selective inhibitor of eIF2α dephosphorylation in INS-1E cells (Cnop, Ladriere et al. 2007) and human islets (Ladrière, Igoillo-Esteve et al. 2010) in an attempt to promote the survival of the clonal β-cells in response to FFA treatment. Unexpectedly, salubrinal induced chronic eIF2α phosphorylation was proapoptotic and potentiated the apoptotic effect of FFA exposure in INS-1E cells and human islets (Cnop, Ladriere et al. 2007, Ladrière, Igoillo-Esteve et al. 2010). This suggests that both the deficiency and chronic activation of eIF2α phosphorylation are poorly tolerated in β-cells and induce the activation of the apoptotic signalling pathways. The apoptotic effect of sustained eIF2α phosphorylation was however found to be restricted to INS-1E cells as other non-β-cell derived cell lines treated with salubrinal were protected against FFA induced apoptosis (Cnop, Ladriere et al. 2007).

Activation of the IRE1 pathway in response to FFA treatment has also been implicated in the propagation of apoptotic signals. Palmitate has been shown to induce the activation of JNK thereby promoting the induction of apoptosis (Cunha, Hekerman et al. 2008). The expression of activated caspase-3 in palmitate treated MEF cells may also be linked to IRE1 through the formation of the IRE1-TRAF2 complex and the activation of the initiator caspase-12 (Tan, Dourdin et al. 2006, Urano, Wang et al. 2000). The overexpression of XBP1s in dispersed rat islets in an attempt to protect β-cells from ER stress induced apoptosis resulted instead in the impairment of GSIS and apoptosis (Allagnat, Christulia et al. 2010).

1.7.4 ER stress in diabetes

Amongst the several mechanisms through which apoptosis may occur, accumulating evidence suggests a role for ER stress in the induction of β-cell apoptosis in diabetes. The most convincing evidence for ER stress mediated β-cell dysfunction and diabetes has been presented in studies in rare genetic disorders.

1.7.4.1 Evidence that ER stress causes β-cell death in humans with diabetes

1.7.4.1.1 Wolcott-Rallison Syndrome

Wolcott-Rallison Syndrome (WRS) is caused by mutations in the EIF2AK3 gene encoding PERK, a transducer of ER stress signalling (Delepine, Nicolino et al. 2000). The mutation occurs in the catalytic domain of PERK resulting in a loss-of-function in its kinase activity and consequently, a reduction in the phosphorylation of eIF2α (Senée, Vattem et al. 2004). The loss of function in PERK kinase activity may result in a disruption in translational attenuation during ER stress, accumulation of misfolded proteins (Liu, Li et al. 2005) and could therefore contribute to β-cell apoptosis (Harding, Zeng et al. 2001). Indeed, autopsy findings in WRS patients include pancreatic hypoplasia and gross loss of β-cells (Thornton, Carson et al. 1997). Evidence for the involvement of PERK in the development of diabetes was presented in study in which mice with a PERK deletion ($PERK^{-/-}$) developed diabetes within a few weeks of birth as a result of progressive β-cell loss (Harding, Zeng et al. 2001, Zhang, McGrath et al. 2002). $PERK^{-/-}$ mice exhibited features reminiscent of the human Wolcott Rallison syndrome including skeletal dysplasia, postnatal growth retardation and exocrine pancreas insufficiency. Mice with a PERK deletion cannot phosphorylate eIF2α and as a result, cannot regulate the translation of insulin in the β-cells (Harding, Zeng et al. 2001). Conditional β-cell specific knockout of PERK by another group also suggested a role for PERK in the foetal and early neonatal development of β-cell mass and function as a prerequisite for postnatal glucose homeostasis (Zhang, Feng et al. 2006). Although some residual PERK expression was detected, the authors of that study suggested that PERK expression in β-cells is not required for the maintenance of β-cell functions and glucose homeostasis in adults. Additionally, mice homozygous for a mutation replacing serine 51 for alanine (Ser51Ala) in eIF2α and resulting in non-phosphorylable eIF2α were reported to die within hours of birth as a result of defective gluconeogenesis (Scheuner, Song et al. 2001). Pancreata from these mice exhibited severe β-cell defects and depletion of insulin at late embryonic and neonatal stages confirming the role of PERK mediated eIF2α in β-cell mass development. While homozygous Ser51Ala mice

die at birth, heterozygous Ser51Ala mice are phenotypically normal but when challenged with a high fat diet, develop glucose intolerance as a result of decreased islet insulin content and a reduction in nutrient induced insulin secretion (Scheuner, Mierde et al. 2005). The β-cells of these mice appear dilated with increased amounts of proinsulin bound to BiP/GRP78, suggesting the accumulation of unfolded/misfolded proteins in the ER (Scheuner, Mierde et al. 2005). All these animal models confirm the observations in the WRS and show that a defect in PERK to eIF2α signalling in β-cells is detrimental to cell survival as such cells cannot balance the translation of insulin with the protein folding capacity within the ER lumen.

1.7.4.1.2 Wolfram Syndrome

Genetic variation in the Wolfram Syndrome gene 1 (WFS1) which encodes an ER Ca^{2+} channel has also been reported to lead to the development of young-onset diabetes known as Wolfram Syndrome (Osman, Saito et al. 2003). Wolfram syndrome is a rare autosomal recessive disorder characterised by diabetes mellitus, optical atrophy and other neurological manifestations (Wolfram, Wagener 1938). The WFS1 gene has been shown to be mutated in 90% of patients with Wolfram syndrome (Khanim, Kirk et al. 2001) and post-mortem studies revealed the selective loss of pancreatic β-cells in subjects with the disease (Karasik, O'Hara et al. 1989). The WFS1 protein is localised to the ER and is highly expressed in the pancreatic β-cell (Fonseca, Fukuma et al. 2005, Takeda, Inoue et al. 2001). The transcriptional and translational upregulation of WFS1 has been reported in response to the activation of PERK and IRE1 under ER stress conditions (Fonseca, Fukuma et al. 2005). Suppression of the gene is however reported to result in the induction of chronic ER stress and subsequently, apoptosis (Fonseca, Fukuma et al. 2005). Mutations in the gene and the evidence for the induction of ER stress in response to the suppression of WFS1 indicates that the pathogenesis of Wolfram syndrome may be attributed to the induction of chronic and unresolved ER stress in the β-cell due to the absence of a functional WFS1 protein. In addition, the expression of WFS1 is also reportedly induced during insulin secretion suggesting a role in the processing and folding of proinsulin within the ER lumen (Lipson K.L et al. 2006, Fonseca, Fukuma et al. 2005). The absence of functional WFS1 protein may

result in the accumulation of unfolded/misfolded proinsulin within the ER lumen and as a result of this lead to the induction of chronic ER stress.

1.7.4.2 Evidence of ER stress in the β-cells of animal models of diabetes

1.7.4.2.1 The Akita mouse

The Akita mouse is a model of non-obese diabetes characterised by a spontaneous mutation in the insulin-2 gene. A missense mutation which replaces a highly conserved cysteine residue at position 96 with tyrosine (*Ins2C96Y)* results in the synthesis of a mutant proinsulin-2 with a defect in the formation of one of the disulphide bonds between the A and B chains (Wang, Takeuchi et al. 1999). The mutant proinsulin accumulates in the ER, is complexed with the chaperone protein BiP/GRP78, induces ER stress and subsequently, apoptosis (Oyadomari, Akio et al. 2002). While the *Ins2* gene is the mouse homologue of the human preproinsulin gene, mice also possess another active insulin gene, *Ins1*. However, despite the presence of normal insulin-1 and of one normal insulin-2 allele in the heterozygous Akita mouse, these animals still develop diabetes as a result of falling insulin production and a progressive decline in β-cell mass due to chronic ER stress induction (Leroux, Desbois et al. 2001). Evidence for ER stress induction in the β-cells of the Akita mouse model includes β-cell distension and the upregulation of the chaperone protein BiP/GRP78 (Araki, Oyadomari et al. 2003). The upregulation of the pro-apoptotic factor, CHOP has also been reported in the β-cells of the Akita mouse (Araki, Oyadomari et al. 2003). Further evidence for the involvement of ER stress induced CHOP expression was shown in a study in which the homozygous disruption of CHOP delayed the development of diabetes and islet cell destruction by 8-10 weeks in the heterozygous Akita mouse (*Ins2C96Y/+)*, suggesting that the induction of apoptosis in only partially dependent on CHOP function (Oyadomari, Akio et al. 2002). The disruption of CHOP in homozygous Akita mice (*Ins2C96Y/C96Y)* was shown to have no measurable effect on the development of diabetes or β-cell death thereby further confirming partial dependency on the expression of CHOP for the destruction of β-cells in the Akita mouse model of diabetes.

1.7.5 ER stress in diabetes and obesity

1.7.5.1 Evidence of ER stress in the islets of humans with type 2 diabetes

Evidence for the involvement of ER stress in human type 2 diabetes was presented in a study which reported a 2-fold increase in the size of the ER in the β-cells of diabetic subjects in comparison to non-diabetics (Marchetti, Bugliani et al. 2007). An increase in ER mass suggests an attempt to increase secretory capacity which may be mediated by the activation of IRE1. Increased immunoreactivity was also observed for ATF3 in pancreatic sections of type 2 diabetic subjects in comparison to non-diabetic subjects (Jiang, Wek et al. 2004, Hartman, Lu et al. 2004). Increased expression of BiP/GRP78 and XBP1 was also observed in the islets of diabetic donors in comparison to non-diabetic donors (Hartman, Lu et al. 2004). In a separate study, increased immunoreactivity was observed for BiP/GRP78 and CHOP in the β-cells from pancreatic tissue sections of type 2 diabetics in comparison to non-diabetics (Laybutt, Preston et al. 2007). The upregulation of CHOP expression has been reported in the β-cells of obese human subjects whether diabetic or not (Huang, Lin et al. 2007). CHOP nuclear localisation was however, observed in the β-cells of obese diabetic patients in comparison to cytoplasmic localisation in obese non-diabetic subjects (Huang, Lin et al. 2007). Although the nuclear localisation of CHOP has not been reported in other studies, this report suggests a role for the nuclear localisation of CHOP in the induction of β-cell apoptosis in type 2 diabetes. It is suggested that the trigger for CHOP nuclear localisation is IAPP as adenovirus-mediated IAPP expression in the INS-1 β-cell line resulted in the upregulation and nuclear localisation of CHOP (Huang, Lin et al. 2007). Amyloid precursor protein was also shown to induce the expression of CHOP in neurons in the absence of an UPR (Copanaki, Schürmann et al. 2007).

1.7.5.2 Evidence of ER stress in the islets of the db/db mouse

The db/db mouse possesses an autosomal recessive mutation in the *db* gene which encodes the leptin receptor (Chua, Chung et al. 1996, Bahary, Leibel et al. 1990). Leptin is a 16-kDa protein which is expressed predominantly in adipose tissue of normal mice (Bjorbaek, Kahn 2004). Leptin is a hormone which functions to reduce energy intake

and expenditure by signalling to the brain and producing a feeling of satiety (Srinivasan K 2007). The lack of functional leptin receptors in the db/db mouse results in hyperphagia, hyperinsulineamia (about 2 wks of age), obesity (which develops at about 4 weeks of age) and insulin resistance. As a result of progressive β-cell failure and apoptosis, the db/db mouse develops hyperglycaemia (at about 4-8 wks of age) and generally does not live for longer than 10 months (Zhou, Berggren et al. 1996, Tuman, Doisy 1977, Bates, Kulkarni et al. 2005). Increased eIF2α phosphorylation, increased expression of ATF4, CHOP, BiP/GRP78, EDEM and XBP1 splicing have been reported in islets isolated from 10-12 wk old diabetic db/db mice in comparison to lean control islets (Laybutt, Preston et al. 2007). While this study addressed the question of ER stress induction in diabetes, it remains to be clarified whether ER stress induction precedes the development of hyperglycaemia in this model of obesity and diabetes. In another study, the upregulation of ATF3 expression was observed in the islets of db/db mice as assessed by real time RT-PCR, indicating the induction of ER stress signalling (Kjørholt, Åkerfeldt et al. 2005).

1.7.6 ER stress as a link between obesity and insulin resistance

Obesity is invariably linked to the development of insulin resistance through the action of high levels of circulating free fatty acids. FFA induced ER stress has been proposed as one of the mechanisms linking obesity with insulin resistance and might therefore represent a common molecular pathway for the two major causes of type 2 diabetes which are insulin resistance and β-cell loss. ER stress and the UPR have been demonstrated to have many links to major inflammatory and stress signalling networks which play significant roles in obesity-induced inflammation and metabolic abnormalities (Deng, Lu et al. 2004, Hu, Han et al. 2006, Hotamisligil 2006). Obesity is associated with low grade inflammation which is causally implicated in the development of insulin resistance. Obesity, which occurs as a result of excess caloric intake, stimulates the synthesis and storage of triglycerides in adipose tissue. The accumulation of fat droplets in adipocytes results in the enlargement of adipose tissue mass as a result of hypertrophy and hyperplasia. Adipocyte hypertrophy results in the release of inflammatory cytokines such as TNF-α, which have been shown to disrupt

insulin action in cells and whole animals (Hotamisligil, Shargill et al. 1993, Uysal, Wiesbrock et al. 1997). In macrophages, experimental induction of the UPR has been shown to stimulate the increased expression of proinflammatory cytokines such as IL-6, MCP-1 and TNF-α (Li, Schwabe et al. 2005), linking ER stress to obesity induced inflammation. ER stress induced as a result of obesity may also trigger the inflammatory signalling pathway through the activation of JNK, which can transcriptionally upregulate several inflammatory genes (Urano, Wang et al. 2000). The suppression of obesity stimulated expression of pro-inflammatory cytokines has been reported in mice lacking JNK resulting in protection from insulin resistance and type 2 diabetes (Hotamisligil, Shargill et al. 1993, Tuncman, Hirosumi et al. 2006). In addition to the upregulation of inflammatory cytokines which are capable of interfering with insulin action, ER stress induced JNK activation has also been implicated in the development of insulin resistance through its inhibitory phosphorylation of IRS1. A role for JNK in the development of insulin resistance was first published by Aguirre et al., in a study in Chinese hamster ovary cells (Aguirre, Uchida et al. 2000). Activated JNK, downstream of IRE1, impairs insulin signalling through the inhibitory phosphorylation of serine residues on IRS-1 and subsequently, a decrease in tyrosine phosphorylation of the insulin receptor and the IRS proteins. Through this process, obesity induced ER stress has been linked to the development of insulin resistance, a major factor in the development of type 2 diabetes. It remains to be determined however, the extent to which obesity induced JNK activation and the metabolic effects on insulin signalling can be attributed to ER stress. Indeed, not all obese insulin-resistant subjects have elevated FFA levels, suggesting that factors other than FFA induced ER stress may contribute to obesity-related insulin resistance and inflammation.

In addition to IRE1 mediated effects, the PERK and ATF6 signalling pathways have also been implicated in the development of insulin resistance. PERK has been reported to mediate the activation of the NF-κB-IKK pathway, which plays a critical role in the induction of multiple inflammatory mediators and has been implicated in the development of insulin resistance (Deng, Lu et al. 2004, Hu, Han et al. 2006). Activated ATF6 has also been linked to NF-κB-IKK signalling (Yamazaki, Hiramatsu et al. 2009),

indicating links between the activation of UPR signalling and the development of insulin resistance.

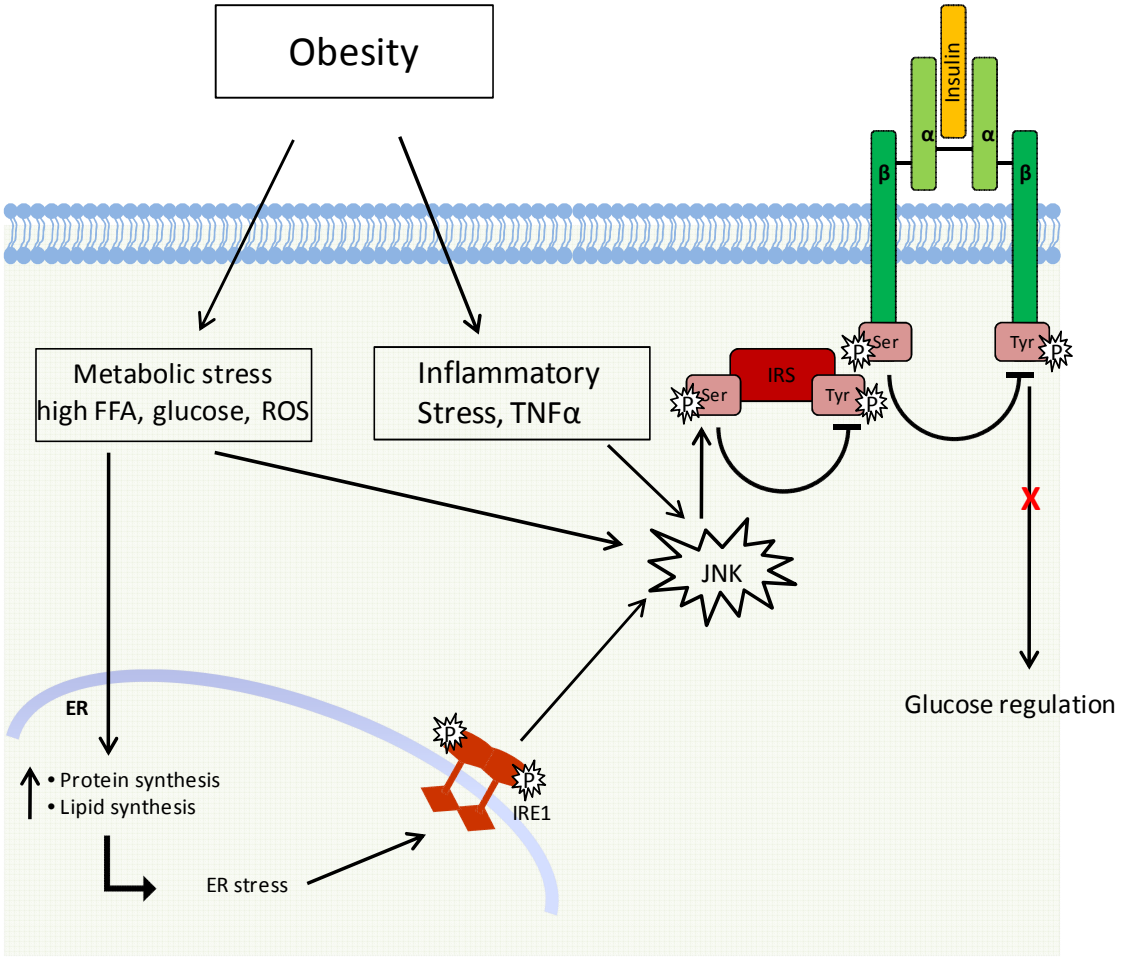


FIGURE 1.9. The role of ER stress in obesity related insulin resistance. Inflammation and metabolic stress induce ER stress and the activation of the UPR. IRE1 activation results in the recruitment of TRAF2 to the ER membrane causing activation of JNK which in turn promotes the inhibitory phosphorylation of serine residues on IRS-1. Serine phosphorylation of IRS-1 inhibits its tyrosine phosphorylation by activated insulin receptors, thus impairing insulin signalling.

1.7.6.1 Evidence of obesity evoked ER stress in the liver

The activation of JNK in response to obesity was first reported in the peripheral tissue of both dietary (high fat fed) and genetic (ob/ob leptin deficient) mouse models of obesity in a study by Hirosumi et al., (Hirosumi, Tuncman et al. 2002). Alterations in the insulin signalling cascade were found to be dependent on the activation of JNK (Hirosumi, Tuncman et al. 2002), a mechanism previously described in CHO cells (Aguirre, Uchida et al. 2000). A separate study by Ozcan et al, also reported the activation of JNK in the liver of the high fat fed and ob/ob mice in comparison to lean controls (Umut, Qiong et al. 2004). In the latter study, other markers of ER stress signalling PERK and eIF2α phosphorylation, upregulation of BiP/GRP78 were also observed in the liver (Umut, Qiong et al. 2004). Similar increases in ER stress markers have also been reported in the livers of obese diabetic db/db mice (Nakatani, Kaneto et al. 2005). The development of insulin resistance and glucose intolerance was greater in high fat fed $XBP1^{+/-}$ mice in comparison to high fat fed $XBP1^{+/+}$ with significant increases in PERK, c-Jun and IRS-1 serine phosphorylation and decreased tyrosine phosphorylation of the insulin receptor, IRS-1 and IRS-2 in the liver (Umut, Qiong et al. 2004).

1.7.6.2 Evidence of obesity evoked ER stress in adipose tissue

ER stress has also been reported in the adipose tissue of high fat fed and ob/ob mouse models of obesity with evidence of PERK phosphorylation, JNK activity and BiP expression in comparison to lean controls (Umut, Qiong et al. 2004). CHOP and BiP expression have also been reported in the adipose tissue of high fat fed mice (Umut, Qiong et al. 2004). In humans, the induction of ER stress was also reported in the adipose tissue of obese insulin-resistant non-diabetic individuals as evidenced by the upregulation of PDI and calnexin in comparison to lean controls (Boden, Duan et al. 2008). The expression of spliced XBP1 and the phosphorylation of JNK in the adipose tissue of the obese subjects are indicative of the development of insulin resistance (Boden, Duan et al. 2008). In a separate study however, the upregulation of ER stress markers such as phosphorylation of eIF2α, expression of BiP, HYOU1 and DNAjc3 was not accompanied by JNK activity (Sharma, Das et al. 2008). A study by Bashan et al.,

also reported on the lack of JNK activity in the subcutaneous adipose tissue (Bashan, Dorfman et al. 2007). Instead, JNK activity was reported in omental adipose.

1.8 Summary and thesis aims

The induction of FFA induced ER stress in the β-cells of human subjects and animal models of obesity and diabetes indicates a role for the signalling pathway in disease pathogenesis and the loss of β-cells. However, how ER stress causes cell death and its contribution to the development of β-cell dysfunction and apoptosis in type 2 diabetes and obesity *in vivo* is unclear. Therefore, the aims of this thesis were to simultaneously:

- Characterise the ER stress to thapsigargin and fatty acids in MIN6 cells
- Determine the patho/physiological role of the PERK pathway in the induction of ER stress mediated apoptosis in MIN6 cells and
- Determine the role of the unfolded protein response in the development of β-cell dysfunction in animal models of obesity and diabetes.

CHAPTER 2

Chapter 2: Materials and Methods

2.1 General Reagents and Materials

Unless stated, all chemicals were of analytical grade and were routinely purchased from Sigma, Fisher or Melford. Unless stated, products for molecular biology were routinely purchased from Promega, Sigma, New England Biolabs or Invitrogen/Gibco BRL. Restriction enzymes and antibodies were purchased from New England Biolabs (NEB). Antibodies were also purchased from Santa Cruz, Abcam and BD Biosciences. RNA extraction kits were purchased from Peqlab and Qiagen. Microfluidic cards containing an array of 48 genes analysed in the animal model study were produced on order from Applied Biosystems (ABI) Foster City, CA 94404. Primers for Taqman™ RT-qPCR were also purchased from Applied Biosystems (ABI). Adenovirus over-expressing GADD34ΔN and DNIRE1α were kindly generated and provided by Dr. Edith Gomez. Disposable plastics (1.5ml microfuge tubes, non-filtered pipette tips) were obtained from Sarstedt. Filtered tips were purchased from Axygen.

2.2 Mammalian Cell Culture

Cell lines used in this study were Mouse Insulinoma 6 cells (MIN6) which respond to physiological changes in glucose concentrations (Miyazaki et al., 1990) kindly provided by Prof. Jun-Ichi Miyazaki, Osaka University Medical School, Japan. Rat Insulinoma-E (INS-1E) cells which are a sub-clone of parental INS1 cells were kindly provided by Prof. Pierre Maechler, Central Medical University, Switzerland (Merglen .A. et al, 2004). Tissue culture plates and flasks were obtained from NUNC or TPP. 96 well plates for luminometry were obtained from Greiner. Tissue culture pipettes were obtained from Greiner or Corning.

2.2.1 Maintenance of Cell Lines

Cells were used at approximately 80% confluence between passages 20 to 50. Mouse Insulinoma 6 (MIN6) cells and INS-1E cells were maintained at 37°C and 5% CO_2 in a humidified incubator. MIN6 cells were cultured in DMEM media containing 25mM glucose supplemented with 15% heat-inactivated FCS, 100µg/ml streptomycin,

100units/ml penicillin sulphate, 100units/ml neomycin, 40mM $NaHCO_3$ and 75µM β-mercaptoethanol. INS-1E cells were cultured in RPMI-1640 containing 11mM glucose supplemented with 5% FCS, 100µg/ml streptomycin, 100units/ml penicillin sulphate, 100units/ml neomycin, 1mM sodium pyruvate, 10mM HEPES and 50µM β-mercaptoethanol. The media was changed in both cell types every 2-3 days.

2.2.2 Cell splitting

When ~80% confluence was reached, media was removed and the cells were washed twice in 1X phosphate buffered saline (PBS; Sigma) before incubation in 1X trypsin/EDTA (Gibco BRL) for 2-5 minutes at 37°C. Cells were resuspended in appropriate media and split 1:3 for maintenance or as required for experiments.

2.2.3 Islet isolation and culture (for Western Blotting)

Pancreatic islets were isolated from 200g to 250g male Wistar rats by collagenase digestion and histopaque density gradient centrifugation by a modification of the method of Guest and Rhodes *et al* (Guest, Rhodes et al. 1989). Briefly, Wistar rats were anesthetized and the pancreas was inflated by injecting 6ml of medium (RPMI 1640 medium containing 11mM glucose) containing 1mg/ml collagenase (Serva, Heidelberg, Germany) through the common pancreatic duct. After the animals were killed by incision of the heart, the pancreas was removed. The excised pancreas was then incubated at 37°C for 17 minutes. Following incubation, the pancreas was vigorously shaken by hand for 1 minute. The partially disaggregated tissue was then centrifuged for 3 minutes at 200 x *g* at 4˚C. The pelleted material was resuspended in RPMI 1640 and subjected to another cycle of re-suspension and centrifugation. The pelleted material was then resuspended in RPMI containing 5% foetal calf serum and then filtered through a 6.5cm-diameter plastic tea strainer. The filtrate was centrifuged for 3 minutes at 200 x *g* at 4˚C and the pellets were resuspended in 10ml of Histopaque 1077 (Sigma) and over-layered with 10ml of RPM1 1640. The tube was centrifuged for 20min at 1600 x *g* at 4˚C and islets were recovered from the RPMI/Histopaque-1077 interface and washed once in RPMI containing 5% foetal calf serum. The islets were then hand-picked under a stereomicroscope to ensure high purity of the preparation. The islets obtained were cultured at 37°C, 5% CO_2, 95% air in RPMI 1640 containing

11mM glucose and 5mg/ml bovine serum albumin instead of 10% foetal calf serum, to prevent flattening of the islets on the bottom of the dish.

Unless otherwise specified, media was changed on the islets the day after isolation and once every other day for a week before experimentation. This was to reduce levels of stress before experimentation.

2.2.4 Islet isolation (for Taqman™ RT-qPCR)

Rats and mice were terminally anaesthetised using rising CO_2 followed by cervical dislocation. They were weighed and the pancreas rapidly removed with the spleen and any adipose tissue attached. The pancreas was placed in cold Krebs' Ringer phosphate HEPES 0.1% BSA (KRH 0.1% BSA) buffer and transferred to the lab on ice. The pancreas was trimmed of fat, spleen and other tissues and then inflated with 5.5 ml cold KRH 0.1% BSA digest cocktail I (see Table below) in a dry petri dish. Leaked cocktail was re-injected into the pancreas and the inflated pancreas transferred into a 50 ml Falcon tube. This was incubated for 3 min in a 37°C water bath while swirling by hand. The tube was then vigorously shaken by hand for 1 min. Incubation in the water bath for 3 min and shaking for 1 min was then repeated. The digest was checked under the microscope to see if it was complete. If digest was incomplete, the volume was made up to 50ml with KRH 0.1% BSA and centrifuged at 950 rpm for 1 min at RT. The pellet was resuspended in cocktail 2 (see table) and incubated at 37°C for 4 min with gentle swirling. This was then manually shaken by hand for 1 min then checked again under the microscope. If digest was still incomplete, it was incubated again for 3 min at 37°C with gentle swirling and shaken by hand for 1 min. The volume was made up to 50 ml with KRH 0.1% BSA and centrifuged at 960 rpm for 1 min at RT. This was repeated 3 X or until the supernatant was clear. The resultant pellet from the digest was resuspended in 20 ml KRH 0.1% BSA and islets picked into clean buffer on ice. A second pick was carried out into clean buffer.

Stock Solutions	Cocktail One		Cocktail Two	
	Add	Final Conc.	Add	Final Conc.
Egg white trypsin inhibitor (200mg/ml)	60µl	1.8mg/ml	50µl	1.5mg/ml
Soybean trypsin inhibitor (200mg/ml)	60µl	1.8mg/ml	50µl	1.5mg/ml
Liberase (10mg/ml)	0.9ml	1.4mg/ml	455µl	0.7mg/ml
Islet Isolation buffer	5.5ml		5.95ml	
Total Volume	6.5ml		6.5ml	

2.2.5 Infection of cell lines with recombinant adenoviruses

Adenoviral mediated transduction of cell lines was performed as follows: Growth medium was aspirated from cells and replaced with 400µl of DMEM (for a 4cm diameter dish) supplemented with 100µg/ml streptomycin, 100units/ml penicillin sulphate, 100units/ml neomycin, 40mM $NaHCO_3$ and 75µM β-mercaptoethanol, minus FCS. High titer or purified viral stock was added to the cells (see figure legends for details) and incubated at 37°C. After one hour, 1.2ml of complete medium (containing heat-inactivated FCS) was added to the cells, and the incubation continued for a further 24-48 h prior to experimentation. Adenoviral infection efficiency was assessed by monitoring the levels of GFP expression using a Nikon fluorescence microscope fitted with a mercury lamp. Efficiency of transduction was typically between 80-95% at the time of experimentation.

2.3 Experimentation

2.3.1 Fatty acid preparation

Free fatty acids oleate and palmitate were purchased from Sigma. 45.67mg of oleate and 41.76mg of palmitate were solubilised in 1ml of 50% ethanol and heated at 50°C to make a 150mM stock solution. Fatty acids were then conjugated to 10% FA-free BSA in a 5:1 molar ratio and left to rock for 1 h at 37°C (Jacqueminet et al., 2000). The solution was filter sterilised and stored in aliquots at -20°C until required. Cells were treated in serum free media with a total fatty acid concentration of 0.5mM and a final unbound free fatty acid concentration in the range of 200nM which is approximately 3 times the unbound concentration in the plasma of lean individuals (Richieri et al., 1993; Richieri et al., 1995). Control conditions contained a similar dilution of FA-free BSA and ethanol to fatty acid preparations.

2.3.2 Treatment of cell lines

Prior to experimentation, cells were washed twice in 1X PBS before the addition of the appropriate media and treatments. Detailed descriptions of treatments are provided in the figure legends. For Western blotting, all treatments were stopped by the addition of ice-cold MIN6 lysis buffer containing 1% Triton, 10mM β-glycerophosphate, 50mM Tris-HCl, pH 7.5, 1mM EDTA, 1mM EGTA, 1mM sodium orthovanadate, 1mM benzamidine HCl, 0.2mM phenylmethylsulfonyl fluoride, 1µg/ml each of leupeptin and pepstatin, 0.1% β-mercaptoethanol, and 50mM sodium fluoride. Lysates were then centrifuged for 10 min at 14 000 rpm (16 000 x *g*). (see section 2.6.3.1). For RNA analysis, treatments were stopped using Trizol reagent (Sigma). (see section 2.4.1).

2.3.3 Determination of cell viability

2.3.3.1 MTT assay

Cells were split 1:3 into 96 well plates (300µl total volume) and left to culture for 48 h. After treatment times elapsed, MTT (5mg/ml) was applied to wells in a 1:10 dilution and incubated for 2 h at 37°C. After this time elapsed, the solution was replaced with 50µl DMSO/well and left to shake at 300 rpm for 5 min at RT. Cell viability was

detected using a BMG lab technologies Novastar plate reader to determine absorbance values at a wavelength of 590nM.

2.3.3.2 FACS analysis

Cells were treated as indicated in figure legends. After experimentation in 4cm dishes (Corning), cells were collected using trypsin and immediately spun down at 900 rpm for 5 min at 4°C. Pelleted cells were resuspended in 100µl of ice cold PBS by gentle pipetting and placed on ice. 900µl of ice cold methanol (Fisher Scientific) was then added in a drop-wise manner. Samples were incubated overnight at -20°C. After overnight incubation, samples were transferred by pipetting into 5ml polystyrene round bottomed tubes (Falcon) and spun down at 900 rpm for 5 min at 4°C. The supernatant was then discarded and the resulting pellet resuspended in 1ml of a solution containing 2µl propidium iodide (PI) (10mg/ml) and 10µl RNase A (1mg/ml) per 1ml of 1X PBS. The mixture was incubated for 30 min at RT and the samples analysed by flow cytometry.

2.4 Molecular Biology

2.4.1 MIN6 RNA extraction (using Tri-Reagent)

Following experimentation, 4cm diameter plates of MIN6 cells were washed once in ice cold 1X PBS then lysed in 500µl of Tri-Reagent (Sigma). Cells were removed from the plate using a cell scraper (Corning) and transferred to a microfuge tube. The tri-reagent/cells mixture was vortexed for 15-30 s and then left at RT for 2 min. 200µl of 1-bromo-3-chloropropane (Sigma) was added and the tube vortexed again for 15-30 s. The tube was incubated at room temperature for a further 5 min to allow dissociation of nucleoprotein complexes. The mixture was then centrifuged at 4°C at 11 800rpm for 15 min in an Eppendorf 5417R refrigerated microfuge using a FA45-30-11 rotor. Following centrifugation, the top aqueous phase (containing RNA) was removed and transferred to a fresh tube. An equal volume of isopropanol was added and the RNA incubated at -80°C for a minimum 30 min period. The precipitated RNA was pelleted by centrifugation at full-speed in a microfuge at 4°C for 10 min and the supernatant discarded. The RNA pellet was washed with 1ml of 70% ethanol, and centrifuged as above for 5 min. The supernatant was discarded and the RNA pellet left to air dry for ~5 minutes at RT. RNA was then resuspended by vigorous vortexing in 25µl distilled water. RNA concentration was determined by diluting 3µl RNA in 597µl ddH_2O and obtaining absorbance values at 260nm and 280nm wavelengths.

2.4.2 MIN6 RNA extraction (using Peqlab RNA extraction kit)

RNA was extracted from MIN6 cells for TaqMan analysis using the Peqlab RNA extraction kit. After experimentation on 4cm dishes, RNA was extracted from cells following manufacturer's instructions. RNA concentration was measured for each sample using a Nanodrop ND-1000 spectrophotometer. 1µl of DNAse1 (RNAse free DNAse set, Qiagen) was added to each sample and the samples were stored at -80°C until required.

2.4.3 Islet RNA extraction (using Qiagen Microeasy kit)

Eppendorfs containing islets were centrifuged at ~12 000 rpm for 1 min and the supernatant discarded. The pellet was resuspended in 500µl 1X PBS and centrifuged

again at ~12 000 rpm for 1 min. The resulting supernatant was discarded and the pellet resuspended in 350µl RLT buffer (Qiagen RNeasy microkit) and stored at -80°C until ready for use. Islets stored in RLT buffer were allowed to defrost and placed on ice. RNA extraction was carried out following the manufacturer's instruction in the Qiagen RNAeasy microkit. RNA concentration (ng/µl) was determined for each sample using a Nanodrop ND-1000 spectrophotometer. 1µl of DNAse1 (RNAse free DNAse set, Qiagen) was added to each sample and the samples were stored at -80°C until required.

2.4.4 Primers

2.4.4.1 XBP1 primers

XBP1 primers were purchased from MWG Biotech AG. The primer sequences are as follows:

XBP1 forward primer	5'–AAACAGAGTAGCAGCCTCAGACTGC-3'
XBP1 reverse primer	5'–TCCTTCTGGGTAGACCTCTGGGA-3'

2.4.4.2 Taqman™ RT-qPCR primers

Pre-complexed primers known as Assay-On-Demand (AOD) sets used for Taqman™ RT-qPCR were purchased on order from Applied Biosystems (ABI) Foster City.

2.4.4.2.1 AOD sets for rat genes

Gene	AOD ID
ATF3	Rn00563784_m1
ATF4	Rn00824644_g1
β2-Microglobulin	Rn00560865_m1
Calreticulin	Rn00574451_m1
Caspase-12	Rn00590440_m1
C/EBPβ	Rn00824635_s1
DDIT3	Rn00492098_g1
EIF4EBP	Rn00587824_m1
ERO1LB	Rn01332228_m1
GADD45A	Rn00577049_m1
HPRT	Rn01527840_m1
HSPA5	Rn00565250_m1
HYOU1	Rn00593982_m1
INS2	Rn01774648_g1
NUCB1	Rn00584973_m1
PDIA4	Rn00587766_m1
RPLP2	Rn01479927_g1

2.4.4.2.2 AOD sets for mouse genes

Gene	AOD ID
ATF3	Mm00476032_m1
ATF4	Mm00515324_m1
β2-Microglobulin	Mm00437762_m1
Calreticulin	Mm00482936_m1
Caspase-12	Mm00438038_m1
DDIT3	Mm00492097_m1
HSPA5	Mm00517691_m1
HYOU1	Mm00491279_m1
PDIA4	Mm00437958_m1
RPLP2	Mm00782638_s1
UCP2	Mm00495907_g1

2.4.5 cDNA synthesis

To prepare cDNA from RNA, 4µg RNA was complexed to 1µl oligo(dT) at 500µg/ml, 0.8µl dNTPs mix (12.5mM) and made up to a 12µl total volume with ddH_2O on ice. The solution was heated at 65°C for 5 min and placed back on ice. The tubes were then spun down and mixed with a solution containing 4µl 5X 1^{st} strand buffer, 2µl 0.1M DTT and 1µl RNA guard. This was gently mixed, spun down and heated at 42°C for 2 min. 1µl of Superscript Reverse Transcriptase (Invitrogen) was then added followed by gentle mixing and a quick centrifugation. The solution was incubated at 42°C for 50 min then heat inactivated at 70°C for 15 min. 1µl of RNaseH (Promega) was added to the solution and incubated at 37°C for 20 min. Synthesized cDNA was stored at -20°C until required for use.

2.4.6 Polymerase chain reaction (PCR)

Taq DNA polymerase (NEB) and specific oligonucleotides were used to amplify specific sequences of XBP1 cDNA. A 50µl reaction mix typically included: 1µl of cDNA, 5µl 10X Pol Mg free buffer, 4µl 25mM $MgCl_2$, 4µl dNTPs mix (2.5mM), 0.5µl each of XBP1 forward and reverse primers, 0.5µl Taq DNA polymerase and the volume was made up to 50µl with ddH_2O. Reaction mixtures were transferred to thin-walled PCR tubes

(ABgene) and were cycled in an Eppendorf Mastercycler Personal thermal cycler. XBP-1 cDNA fragments were amplified using the following PCR reaction programme:

94°C	3 min	
94°C	30 s	31X
55°C	30 s	
72°C	1 min	
72°C	5 min	
4°C	End	

An initial denaturing period of 94°C for 30 sec was followed by annealing at 55°C for 30 s, and an extension period at 72°C for 1 min for every 1-kb to be amplified. This cycle was repeated x31 times. There was a final 5 min extension period at 72°C and then the reaction mixture was kept at 4°C. PCR reactions were analysed by agarose gel electrophoresis. 5µl of the PCR product was loaded onto a 3% agarose gel in 1X TAE buffer with ethidium bromide to determine that the appropriate fragment had been generated.

2.4.7 TAE-agarose gel electrophoresis

Agarose (0.7%-1.5%) (Melford) was dissolved in 1X TAE by heating in a microwave for ~2 min or until the powder had fully dissolved. Once cooled to ~60°C, 0.1µg/ml ethidium bromide was added and the gel allowed to set in a pre-cast tray. DNA samples were prepared by the addition of DNA loading buffer and samples were loaded alongside 100bp DNA ladders (NEB). Agarose gels were run horizontally immersed in 1X TAE at 90 volts for ~1 h or as required for the resolution of bands.

2.4.8 Ethanol precipitation of DNA

40µl of PCR product was precipitated with 80µl 100% ethanol and 4µl 3M NaOAc (pH 5.2). The solution was left at -20°C for 1 h and spun down at 14 000 rpm at 4°C for 10 min. The supernatant was removed and the resulting pellet washed with 750µl 70% ethanol and spun down at 14 000rpm at 4°C for 10 min. The supernatant was then discarded and the DNA pellet air dried and resuspended in 20µl ddH_2O.

2.4.9 Restriction digestion of DNA

Restriction endonucleases were obtained from New England Biolabs. 5µl of the resulting cDNA was incubated with 1µl of the restriction enzyme Pst1, 2µl NE Buffer 3 10X, 0.2µl BSA 100X and 11.8µl ddH_2O (total volume 20µl) for at least 1.5 h at 37°C. Restriction digests were prepared by the addition of loading buffer and separated on 3% agarose gel with ethidium bromide. The gels were photographed under UV illumination using a Flowgen UV illuminator connected to a Canon 7-21mm 1:2.0-2.5 zoom lens camera.

2.4.10 Taqman™ RT-qPCR

TaqMan™ RT-qPCR was used to determine the relative expression of genes using 'Assay-On-Demand' (AOD) primers and probes (Applied Biosystems). All islet RNA samples were normalised to 10ng/µl for TaqMan analysis. Using the TaqMan™ RNA-to-C_T 1-step kit (Applied Biosystems), each 10µl reaction comprised of 2µl of normalised RNA, 0.25µl TaqMan RT Enzyme Mix (40x), 5µl TaqMan RT-PCR Mix (2x), 0.5µl AOD and 2.25µl RNAse free water. All samples were run in triplicate on a Taqman 7900/7700 machine. Reactions without reverse transcriptase were also compiled for each sample to control for genomic DNA contamination. A pool of RNA samples normalised to 50ng/µl was serially diluted and run alongside each sample to generate a standard curve. The average of triplicate Taqman qRT-PCR reactions for each gene was normalised to the average of triplicates for housekeeping gene 18s or RPP2. Data are represented as a mean of animals in each group ± SEM.

2.5 Recombinant adenoviral techniques

2.5.1 Harvesting virus from HEK-293 cells

Viruses were ready for harvesting 7 to 10 days post-transfection of the recombinant adenoviral plasmids. Cells were washed off the flasks, transferred to 50ml conical tubes and pelleted at 1 800 x *g* for 5 min at 4°C in an Eppendorf 5810R centrifuge. The pellets were resuspended in 1ml PBS, frozen in a dry ice/ethanol bath then thawed in a 37°C water bath and vortexed. This freeze/thaw/vortex cycle was repeated 3 more times to fully lyse the cells. The samples were spun again at 3 200 x *g* for 10 min at 4˚C to pellet cell debris. The pellet was discarded and the resulting viral supernatant was stored at -80°C.

2.5.2 Generation of high titre adenovirus stocks

90% of the adenoviral supernatant was used to re-infect two confluent T-25 flasks of HEK-293 cells. When GFP was present, transfections and viral productions were monitored by GFP expression on a Nikon fluorescence microscope fitted with a mercury lamp. Approximately 3-5 days post-infection, when 70-90% of the cells were floating, the cells were harvested and subjected to four cycles of freeze/thaw/vortex as described above. The virus supernatant from two T-25 flasks was used to infect two T-75 flasks. Supernatant from this round of amplification was then used to infect 5 T-75 flasks. Finally high titre virus was produced when the virus supernatant from 5 T-75 flasks was used to infect 20 T-75 flasks.

2.6 Protein Techniques

2.6.1 Buffers and reagents

Lysis Buffer

1% v/v Triton X-100

10mM β-glycerophosphate pH 7.4

50mM Tris-HCl pH 7.5

1mM EDTA pH 8

1mM EGTA

1mM Sodium Orthovanadate

1mM Benzamidine

0.2mM PMSF

1µg/ml Leupeptin

1µg/ml Pepstatin A

0.1% v/v β-mercaptoethanol

50mM NaF

10X Tris-Glycine buffer (for 1l)

30g Tris base (Melford)

144g Glycine (Melford)

Krebs' Ringer Phosphate Buffer (Islet Isolation buffer)

129mM NaCl

5mM $NaHCO_3$

4.8mM KCl

1.2mM KH_2PO_4

1.2mM $MgSO_4$

10mM HEPES

pH to 7.4 with 1M NaOH then add:

2.5mM $CaCl_2$

5.6mM Glucose

0.1% BSA

SDS-PAGE Running Buffer

1X Tris-Glycine buffer

0.1% w/v SDS

Semi-Dry Transfer Buffer

1X Tris-Glycine buffer

0.01% w/v SDS

20% v/v Methanol

10X PBS (Phosphate Buffered Saline; 1l)

3g KCl

100g NaCl

14g Na_2HPO_4

3g KH_2PO_4

pH to 7.4 and make up to 1 litre with distilled water

PBS-Tween (PBST)

1X PBS

0.1% v/v Tween-20 (Sigma)

Laemmli Sample buffer (4X)

0.25M Tris pH 6.8

4% w/v SDS

40% v/v Glycerol

10% v/v β-mercaptoethanol

20μg/ml Bromophenol blue

2.6.2 Antibodies

Antibody	Obtained from	Catalog #	Primary Dilution	Secondary Antibody	Application
ATF4	Santa Cruz	SC-200	1:1000	Rabbit	WB
ATF6	Imgenex	IMG-273	1:1000	Mouse	WB
Calreticulin	*Sigma*	*C4606*	1:20000	Mouse	WB
Cleaved Caspase3	NEB	9664	1:500	Rabbit	WB
p-c-Jun	NEB	9261	1:1000	Rabbit	WB
CPE	BD Transduction	610758	1:1000	Mouse	WB
p-eIF2α	Biosource	44-728G	1:1000	Rabbit	WB
p-eIF2α	NEB	9721	1:1000	Rabbit	WB
eIF2α	Santa Cruz	sc-11386	1:1000	Rabbit	WB
eIF2α	NEB	9722	1:1000	Rabbit	WB
GADD153 (CHOP)	Santa Cruz	sc-793	1:200	Rabbit	WB
GAPDH	Abcam		1:10000	-	WB
GAPDH	Santa Cruz	sc-25778	1:1000	Rabbit	WB
GFP			1:1000	Mouse	WB
GRP78 (BiP/HSPA5)	BD Transduction	610978	1:1000	Mouse	WB
GRP94	Abcam	Ab13509	1:1000	Rabbit	WB
HYOU1 (ORP150)	Santa Cruz	sc-136555	1:5000	Mouse	WB
p-IRE1α	Novus Biologicals	NB 100-2323	1:1000	Rabbit	WB
IRE1α	Novus Biologicals	NB 100-2324	1:1000	Rabbit	WB
p-PERK (Thr 980)	NEB	3191	1:500	Rabbit	WB
XBP1	Santa Cruz	sc-7160	1:500	Rabbit	WB
4EBP1	NEB	9644B	1:1000	Rabbit	WB

2.6.3 Sample preparation

2.6.3.1 Protein extraction from cells

Following experimentation, cells were scraped off 4cm diameter plates in ~110µl of ice cold MIN6 lysis buffer and transferred to a clean microfuge tube. The whole cell lysate was vortexed for 10 s and centrifuged at 14 000 rpm for 10 min at 4°C. The supernatant was removed and transferred to a fresh microfuge tube. To obtain nuclear fractions, cells were briefly washed with ice-cold PBS then collected in 1X PBS in microfuge tubes. After spinning at 500 x *g* for 2-3 min at 4°C, the supernatant was discarded leaving the pellet as dry as possible. Nuclear extracts were obtained following instructions for 20 µl packed cell volume in the NE-PER nuclear and cytoplasmic extraction reagents kit (Thermo Scientific). Protein concentration in lysates was determined by Bradford assay and protein expression analysed by Western blotting. Samples were stored at -20°C until required.

2.6.3.2 Protein extraction from tissues

Approximately 50mg of tissue was placed in round bottom tube on ice and homogenised in 1ml of RIPA buffer containing protease inhibitors. Samples were sonicated and spun down in a micro-centrifuge at 14 000 rpm for 10 min at 4°C. Supernatant was removed and transferred to a new Eppendorf tube. Protein concentration in samples was determined by Bradford and protein expression was analysed by Western blotting. Samples were stored at -80°C until required.

2.6.4 Bradford assay

The Bradford protein assay was used to determine the protein content of cell and tissue lysates. Bradford reagent (Bio-Rad) was diluted with ddH_2O in a 1:5 ratio for use. 2µl of protein samples of unknown protein content with 8µl ddH_2O was mixed with 1ml of diluted Bradford reagent and incubated for 5 minutes at RT. The light absorbance of the reaction mixture at a wavelength of 595nm was measured on a WPA spectrophotometer, and the protein content of unknowns determined by linear regression against a standard curve of BSA protein standards (0 to 20µg). Protein contents of individual lysates were adjusted to the sample with the lowest protein content using the same lysis buffer used in the experiment. Samples were complexed to 4X Laemmli sample buffer in a 3:1 ratio and boiled for 3 min at 100°C before running on an SDS-PAGE gel.

2.6.5 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels

2.6.5.1 Gel formulation

The running gel was poured between glass plates and then immediately overlaid with distilled water. After the gel has set (~30 minutes), the water was removed by decantation and the stacking gel immediately poured on top. The comb was inserted immediately after and the gel left to set for ~30 minutes.

SDS-PAGE (Enough for two ATTO system running gels and 4 stacking gels)

SOLUTION	7.5%	10%	12.5%	15%	17.5%	20%	STACKING
40% Acrylamide(BDH)	2.925ml	3.825ml	4.725ml	5.625ml	6.525ml	7.275ml	1.24ml
2% Bis-Acrylamide[1]	1.56ml	2.04ml	2.52ml	3ml	3.48ml	3.75ml	0.65ml
1.5M Tris-HCl, pH 8.8	3.75ml	3.75ml	3.75ml	3.75ml	3.75ml	3.75ml	-
1M Tris-HCl, pH 6.8	-	-	-	-	-	-	1.25ml
H_2O	6.54ml	5.165ml	3.78ml	2.4ml	1.02ml	0ml	6.7ml
10% SDS	0.15ml	0.15ml	0.15ml	0.15ml	0.15ml	0.15ml	0.1ml
TEMED[2]	7.5µl	7.5µl	7.5µl	7.5µl	7.5µl	7.5µl	10µl
10%APS[3] (Add just before pouring)	75µl	75µl	75µl	75µl	75µl	75µl	75µl

Acrylamide: Bis acrylamide ratio of 37.5:1

1: N,N'-methylene bis-acrylamide (BDH/Bio-Rad)

2: NNN'N' tetramethylethylenediamine

3: Ammonium persulphate

2.6.5.2 Running of gels

SDS-PAGE gels were immersed in SDS-PAGE running buffer in ATTO system gel tanks. Protein samples (prepared as described in section 2.6.4) were boiled for 3 min at 100°C, then centrifuged briefly at full speed and loaded onto SDS-PAGE gels alongside prestained protein markers (broad range; NEB). SDS-PAGE gels were run vertically at 180 volts for ~90 min or until the bromophenol blue had run off the edge of the gel.

2.6.6 Western blotting

Protein samples were run on SDS-PAGE gels as described above. Proteins were transferred onto Immobilon-P PVDF filters (Millipore) using a Semi-Dry Transfer Cell (Bio-Rad) or wet transfer tank (Bio-Rad). For semi-dry transfers, membranes were soaked in 100% methanol to wet them. 6 Whatmann 3MM papers per gel, the membrane and the gel were equilibrated in semi-dry transfer buffer for 5 min. 3 Whatmann papers were placed in the cell and air bubbles removed. The membrane was then layered onto the papers. The gel was placed on top of the membrane and 3 more Whatmann papers placed on top. Any air bubbles and excess transfer buffer were removed. The gel was then blotted at 15 volts for 38 min. For wet transfers, membranes were also soaked in 100% methanol to wet them. 2 Whattmann 3MM papers per gel, the membrane and the gel were equilibrated in transfer buffer for at least 5 min. One Whatmann paper was placed on a foam based cell and air bubbles removed. The gel was placed on the paper, followed by the membrane and one more Whatmann paper placed on top. Foam was placed on top of the stack and the stack held firmly together in a case. The gel was blotted for 100 volts for 1 h in a -4°C cold room. Transfer efficiency was assessed by transfer of the prestained protein markers. Following transfer, the membrane was blocked in 5% milk PBST for 1 h at RT. Where antibodies were diluted in milk, the blocking step was followed by an overnight incubation at 4°C in the specific antibody. Where primary antibodies were diluted in 5% BSA PBST, the membrane was washed 3X for 5 min each in PBST prior to overnight incubation. Antibody concentrations were determined according to manufacturer recommendations. The following day, the membrane was washed 3X for 10 min each with PBST. The membrane was then incubated in the appropriate secondary antibody (secondary antibodies from NEB was diluted 1:3000 and made in 5% milk PBST) for 1 h at RT. Detection of proteins was performed by enhanced chemiluminescence (ECL, Amersham Biosciences) of membranes to X-ray film (GRI) for 1 min to 1 h depending on protein signal. Proteins of interest were identified by comparison of their size to broad range protein markers.

2.6.7 Quantification and statistical analysis

Statistical differences between multiple groups were analysed via single factor analysis of variance (ANOVA) followed by a Bonferroni post-test. Comparison between two sets of data was analysed using an unpaired two-tailed Student's t-test. A statistical test was only carried out when the experiments had at least n=3. Statistical significance was only confirmed when $p \leq 0.05$. Statistical analyses were carried out using the GraphPad Prism software (GraphPad Prism, San Diego, CA).

CHAPTER 3

Chapter 3: Characterisation of the ER stress response in mouse insulinoma (MIN6) cells

3.1 Introduction

Type 2 diabetes results from the inability of the pancreatic β-cells to compensate for insulin resistance in peripheral tissues (Poitout, Robertson 1996). This failure to compensate for insulin resistance occurs due to defects in insulin secretion by the β-cell and/or the loss of β-cell mass as a consequence of cell death (Butler, Janson et al. 2003a, Porte, Kahn 2001). Elevated levels of circulating free fatty acids (FFAs) which may be present in obese individuals, have been identified as a major contributor in the development of insulin resistance in peripheral tissues and the induction of β-cell death in type 2 diabetes (Donath, Halban 2004). *In vitro,* FFA induced defects in β-cells have been reported to mimic the β-cell defects observed in type 2 diabetics including an increase in basal insulin secretion, impaired response to glucose and the induction of apoptosis (Shimabukuro, Zhou et al. 1998, Zhou, Grill 1994, Busch, Cordery et al. 2002). Evidence of FFA cytotoxicity in β-cell lines and isolated rat islets has also been published (Shimabukuro, Zhou et al. 1998, Cnop, Hannaert et al. 2001, Maedler, Spinas et al. 2001, Carlsson, Hakan Borg et al. 1999). While the exact mechanism(s) by which fatty acids induce apoptosis is currently unknown, several mechanisms have been implicated including the formation of triacylglycerols (TAG), ceramide synthesis, formation of nitric oxide and reactive oxygen species (ROS) and ER stress (Karaskov, Scott et al. 2006, Moffitt, Fielding et al. 2005, Kelpe, Moore et al. 2003, Maestre, Jordan et al. 2003).

Long chain saturated fatty acids such as palmitate (C16:0) and stearate (C18:0) have been shown to be toxic to β-cells (Cunha, Hekerman et al. 2008, Maedler, Spinas et al. 2001, Lai, Bikopoulos et al. 2008, El-Assaad, Buteau et al. 2003), while long chain unsaturated fatty acids such as palmitoleate (C:16:1) and oleate (C18:1) and short chain saturated fatty acids such as myristate (C14:0) and laurate (C12:0) are reported to be cytoprotective or much less toxic than saturated fatty acids (Diakogiannaki, Welters et al. 2008, Welters, Tadayyon et al. 2004, Diakogiannaki, Dhayal et al. 2007,

Dhayal, Welters et al. 2008). There are however, conflicting reports on the effect of the unsaturated fatty acid oleate on β-cell apoptosis. While the general consensus is for its protective effects on cell survival, several studies have reported that oleate can induce apoptosis (Kharroubi, Ladriere et al. 2004, Cunha, Hekerman et al. 2008, Karaskov, Scott et al. 2006). The differential effect of saturated *versus* unsaturated fatty acids on apoptosis induction has been suggested to be due to the greater propensity of unsaturated fatty acids to form intracellular triglycerides (TAG) (Briaud, Harmon et al. 2001, Unger, Zhou 2001, Unger, Orci 2002). Conversion of FFA into TAG has been reported to be cytoprotective in some studies (Cnop, Hannaert et al. 2001, Listenberger, Han et al. 2003, Busch, Gurisik et al. 2005) while others have shown evidence of cytotoxicity (Moffitt, Fielding et al. 2005, Briaud, Harmon et al. 2001). It is suggested however, that TAG accumulation may not play a significant role in the induction of β-cell apoptosis as treatment of rat β-cells with bromopalmitate, a non-metabolizable analogue of palmitate, was reported to be cytotoxic, even in the absence of TAG accumulation (Cnop, Hannaert et al. 2001). In addition, a more recent study suggests that the cytotoxic effects of fatty acids vary even when TAG formation is unaltered (Diakogiannaki, Dhayal et al. 2007). The ability of FFAs to be stored as TAGs and evidence for the induction of apoptosis in response to bromopalmitate suggests that TAG formation may not play a major role in FFA induced β-cell apoptosis.

The *de novo* synthesis of potentially toxic ceramide from the by-products of palmitate metabolism has also been suggested to mediate apoptosis induction in β-cells while unsaturated fatty acids do not induce ceramide synthesis (Holland, Summers 2008). The promotion of ceramide synthesis was observed when β-cells were exposed to palmitate (Kelpe, Moore et al. 2003), and treatment of islet cells with ceramide analogues has been reported to induce the loss of cell viability (Shimabukuro, Higa et al. 1998; Veluthakal, Palanivel et al. 2005). There is however, no general consensus that palmitate mediates its apoptotic effects through ceramide synthesis as an inhibitor of ceramide synthesis, fumonisin B1, was unable to inhibit palmitate induced apoptosis in BRIN-BD11 and RINm5F insulin producing beta cell lines (Welters, Tadayyon et al. 2004; Beeharry, Chambers et al. 2004). In addition, the non-metabolizable palmitate analogue, bromopalmitate, was found to be cytotoxic even in

the absence of its metabolism in rat β-cells, suggesting that ceramide was not required for toxicity (Cnop, Hannaert et al. 2001). More recently however, a study reported that the inhibition of ceramide synthesis in MIN6 cells exposed to palmitate for 48 h resulted in a decrease in ER stress and apoptosis (Boslem, Macintosh et al. 2011). These conflicting reports suggest that the contribution of ceramide synthesis to palmitate induced apoptosis in β-cells requires further investigation.

The upregulation of an inducible form of NO synthase (iNOS) mRNA expression and the generation of nitric oxide (NO) in response to palmitate treatment has also been implicated in β-cell death (Shimabukuro, Ohneda et al. 1997). Others have however failed to identify a role for NO synthesis in palmitate induced apoptosis in isolated human islets or rat β-cells (Cnop, Hannaert et al. 2001, Lupi, Dotta et al. 2002).

The generation of reactive oxygen species (ROS) in response to palmitate treatment has also been implicated in the induction of β-cell apoptosis (Carlsson, Hakan Borg et al. 1999). Conflicting evidence against the involvement of ROS in palmitate induced apoptosis has however, also been published (Cnop, Hannaert et al. 2001, Lupi, Dotta et al. 2002, Shimabukuro, Ohneda et al. 1997). It appears that while these factors may be involved in the induction of palmitate mediated β-cell death, the extent of their involvement may be minimal and/or inconsequential to the main mechanisms involved in β-cell death.

In addition, the activation of a specific isoform of protein kinase C (PKC) has also been implicated in FFA induced β-cell death. PKCδ is reported to be required for apoptosis in several cell lines (Denning, Wang et al. 1998, Reyland, Anderson et al. 1999, Choi, Hur et al. 2006) and palmitate exposure was reported to induce PKCδ nuclear translocation as an early event in insulin producing β-cell lines (Eitel, Staiger et al. 2003, Alcazar, Qiu-yue et al. 1997). These studies were carried out in the presence of rottlerin, a reportedly specific PKCδ inhibitor (Gschwendt, Muller et al. 1994) and inhibitor of mitochondrial metabolism which induces collapse of the mitochondrial membrane potential (Liao, Hung et al. 2005, Ringshausen, Oelsner et al. 2006). The potential involvement of PKCδ in palmitate mediated apoptosis was also reported in INS-1 cells (Wrede, Dickson et al. 2003) and in β-cells expressing a kinase negative

form of PKCδ (Hennige, Ranta et al. 2010). In contrast, a study using the PKC activator, phorbol-12-myristate-13-acetate (PMA) reported that the activation of PKCδ had no effect on palmitate induced apoptosis in BRIN-BD11 cells (Welters, Smith et al. 2004). Furthermore in that study, the downregulation of PKCδ did not prevent palmitate induced apoptosis in the BRIB-BD11 cell line. Although the report by Hennige at al., provides substantial evidence for the involvement of PKCδ in palmitate induced apoptosis *in vivo*, a general consensus on the role of PKCδ in palmitate induced apoptosis has not been reached.

In addition to the mechanisms outlined above, palmitate induced ER stress has also been implicated in the induction of β-cell death in type 2 diabetes. Evidence for ER stress induction in type 2 diabetes includes the expression of ER stress markers such as BiP and pro-apoptotic CHOP in human diabetic pancreatic sections (Laybutt, Preston et al. 2007). The upregulation of ER stress and apoptotic markers have also been shown in β-cell lines chronically exposed to palmitate (Kharroubi, Ladriere et al. 2004, Laybutt, Preston et al. 2007, Karaskov, Scott et al. 2006). In some studies, the unsaturated fatty acid oleate was also reported to induce ER stress and subsequently apoptosis (Kharroubi, Ladriere et al. 2004, Cunha, Hekerman et al. 2008). Apoptosis, as a result of palmitate induced ER stress, occurs via signalling events downstream of the ER stress transducer proteins. Chronic palmitate induced PERK activation results in the upregulation of a pro-apoptotic transcription factor, CHOP (Harding, Novoa et al. 2000b). The exact mechanism(s) by which CHOP induces apoptosis is unknown. It has however been shown to induce the upregulation of ERO1α (Marciniak, Yun et al. 2004), an ER oxidase which may induce hyperoxidising conditions within the ER thereby increasing the levels of unfolded proteins and ER stress (Lai, Teodoro et al. 2007). The upregulation of GADD34 downstream of CHOP has also been implicated in ER stress induced apoptosis (Novoa, Zeng et al. 2001). GADD34 promotes the dephosphorylation of eIF2α thereby mediating the recovery of protein translation. Under conditions of ER stress, the recovery of translation will result in an increase in protein load within the ER lumen thereby enhancing ER stress (Ron 2002). CHOP also downregulates the expression of the anti-apoptotic protein Bcl-2 and increases cellular ROS thus contributing to the induction of ER stress mediated cell death (Heather,

Yuhong et al. 2003, McCullough, Martindale et al. 2001). CHOP activity is however not the only mechanism through which ER stress induced apoptosis is mediated. Studies in PERK KO and eIF2α (S51A) mutant cells provided evidence of the induction of apoptosis in response to ER stressors in the absence of CHOP induction (Heather, Yuhong et al. 2003, Scheuner, Song et al. 2001). The disruption of the CHOP gene in heterozygous Akita mice also only resulted in a delay but not complete inhibition of ER stress induced β-cell apoptosis (Oyadomari, Akio et al. 2002). Other signalling mechanisms must therefore play a role alongside CHOP in the propagation of ER stress induced apoptosis.

Palmitate induced IRE1α activation has also been implicated in the induction of apoptosis through the activation of JNK signalling. Activated IRE1α recruits the adaptor protein TNF receptor-associated factor 2 (TRAF2) to the ER membrane (Urano, Wang et al. 2000) thereby forming a complex to which the apoptosis signal-regulated kinase 1 (ASK1) is recruited. Activated ASK1 induces the activation of JNK and subsequent signalling downstream of JNK (Urano, Wang et al. 2000, Nishitoh, Saitoh et al. 1998). It has been suggested that JNK mediates its apoptotic effects downstream of IRE1α through the phosphorylation and subsequent inactivation of the Bcl-2 family of proteins (Yamamoto, Ichijo et al. 1999). Activation of the caspase cascade may also be induced by TRAF2 mediated caspase-12 activation (Yoneda, Imaizumi et al. 2001).

The initial protective nature of the UPR therefore appears to switch into apoptotic signals through an unknown mechanism. ER stress induction also results in changes in the expression of genes which may be involved in apoptotic signalling. Studies using proteomic screenings have identified changes in the expression of several candidate genes in response to palmitate exposure (Busch, Cordery et al. 2002, Biden, Robinson et al. 2004). Indeed, a study using inhibitors of gene transcription and mRNA translation reported marked attenuation in the ability of saturated fatty acids to induce β-cell death (Welters, McBain et al. 2004). This suggests that early changes in gene expression in response to fatty acid treatment may be involved in the induction of apoptotic signals. Treatment of isolated human islets with palmitate was reported to result in the downregulation of the expression of Bcl-2, an anti-apoptotic protein expressing gene (Lupi, Dotta et al. 2002). While the mechanisms remain to be clarified,

the induction of β-cell apoptosis by palmitate and in some cases, oleate appears to be important in the pathogenesis of type 2 diabetes.

3.1.1 Aims

The relevance of ER stress in the development of obesity associated type 2 diabetes is not fully understood. In this study, I set out to characterise ER stress signalling and cell survival in response to thapsigargin, a pharmacological inducer of ER stress, as well as saturated *versus* unsaturated fatty acids in the mouse insulinoma (MIN6) clonal pancreatic β-cell line (Miyazaki, Araki et al. 1990). The insulin-secreting glucose responsive MIN6 cell line was used for this study as a source of highly differentiated and homogenous β-cells (Miyazaki, Araki et al. 1990, Webb, Akbar et al. 2000).

3.2 Results

3.2.1 Thapsigargin induces time-dependent ER stress signalling in MIN6 cells

3.2.1.1 Activation of the UPR

Thapsigargin induces ER stress by inhibiting the function of the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) pump on the ER membrane (Lytton, Westlin et al. 1991). Inhibition of the SERCA pump results in the depletion of calcium levels within the lumen of the ER. This leads to a disruption of homeostatic balance within the ER lumen and as a consequence, the accumulation of unfolded proteins resulting in ER stress. MIN6 cells were treated with thapsigargin for up to 48 h to induce ER stress. Changes in the phosphorylation state and expression of markers of ER stress and the UPR were determined by Western blotting. Thapsigargin induced PERK phosphorylation by 2 h and this was sustained up to 48 h (**Figure 3.1ai**). The phosphorylation of eIF2α, a downstream target of PERK, was also detected by 2 h and peaked at 12 h. This was followed by a progressive decline up to 48 h of thapsigargin treatment (**Figure 3.1ai, aii**). The phosphorylation of eIF2α results in the attenuation of global translation within the cell, a protective feature of the UPR signalling (Harding, Zhang et al. 1999). p-eIF2α is however reported to induce a paradoxical increase in ATF4 translation which stimulates the expression of proteins required for recovery from ER stress (Harding, Novoa et al. 2000b). Indeed, an increase in the expression of ATF4 was observed by 2 h of thapsigargin treatment followed by a progressive decline back to basal levels by 48 h of exposure (**Figure 3.1ai**). The rate of ATF4 translation was also assessed in our cells by measuring the expression of luciferase in MIN6 cells infected with adenovirus expressing luciferase downstream of the 5' untranslated region (UTR) of ATF4 (Ad-ATF4luc). While thapsigargin induced ATF4 protein expression was evident by Western blotting from 2 h of treatment (**Figure 3.1ai**), increased luciferase expression was only apparent from 12 h of thapsigargin treatment (**Figure 3.1aiii**). The reasons for this difference are unclear.

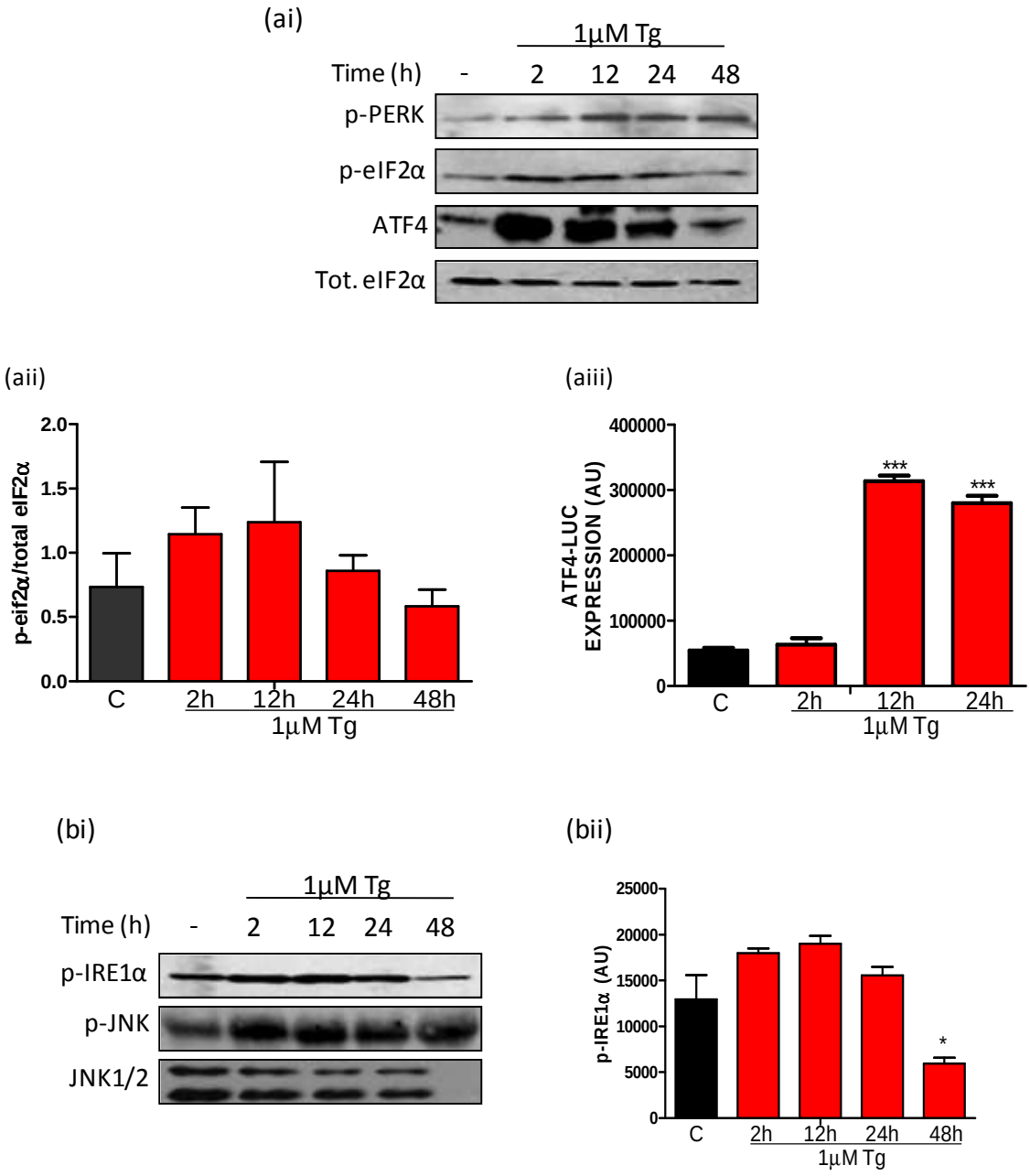
Thapsigargin treatment also induced IRE1α phosphorylation in MIN6 cells from 2 h of exposure and peaked at 12 h (**Figure 3.1bi**). A significant decline in the

phosphorylation of IRE1α below basal levels was observed by 48 h of thapsigargin exposure (**Figure 3.1bi, bii**). XBP1 splicing was also assessed by reverse transcriptase-PCR of the XBP1 mRNA followed by digestion with the restriction enzyme; Pst1. PCR products and the products of this reaction were run out on a 3% agarose gel and the spliced and unspliced forms of XBP1 detected based on differences in gel mobility when subjected to electrophoresis. Unspliced XBP1 retains the restriction site and is cleaved into two smaller fragments of 290 and 190 base pairs. The excision of a 26-nucleotide intron from XBP1 yields the spliced form of XBP1 made up of 454 base pairs. Thapsigargin induced XBP1 splicing was evident from 12 to 24 h and a decline was observed by 48 h (**Figure 3.1c**). A decrease in XBP1 splicing at 48 h correlated with the decline in the phosphorylation status of IRE1α. In addition to its role in the splicing of XBP1, activated IRE1α has also been implicated in the downstream phosphorylation and activation of JNK signalling under ER stress conditions (Nishitoh, Saitoh et al. 1998, Nishitoh, Matsuzawa et al. 2002). Thapsigargin induced JNK phosphorylation from 2 h of exposure in MIN6 cells and this was sustained for the duration of the experiment (**Figure 3.1bi**).

The activation of the IRE1α and ATF6 arms of the UPR is reported to mediate the upregulation of chaperone proteins and foldases (Lee, Tirasophon et al. 2002, Yamamoto, Yoshida et al. 2004). In our MIN6 cells, thapsigargin induced a small but insignificant increase in GRP78/BiP expression (**Figure 3.1d**) but no change was observed in the expression of other chaperone proteins such as GRP94, HYOU1 and calreticulin (**Figure 3.1d**). ER stress, specifically in response to the saturated fatty acid palmitate, has also been reported to induce the degradation of carboxypeptidase E (CPE), an essential enzyme in the production of multiple peptide hormones including insulin (Jeffrey, Alejandro et al. 2008, Johnson 2009). No change was however detected in the expression of CPE in MIN6 cells in response to thapsigargin treatment (**Figure 3.1d**).

The upregulation of ATF4 expression has been reported to stimulate the expression of 4EBP1, a suppressor of the mRNA 5' cap-binding protein eukaryotic initiation factor 4E (Novoa, Zeng et al. 2001, Okada, Yoshida et al. 2002, Suguru Yamaguchi, Hisamitsu Ishihara et al. 2008). In this study, thapsigargin also induced the expression of 4EBP1

from 12 h of exposure (**Figure 3.1d**). The increase in 4EBP1 expression was sustained up to 24 h and decreased back to basal levels after 48 h of thapsigargin exposure (**Figure 3.1d**).


(ai)
1µM Tg
Time (h) - 2 12 24 48
p-PERK
p-eIF2α
ATF4
Tot. eIF2α
(aii)
p-eif2α/total eIF2α
2.0
1.5
1.0
0.5
0.0
C 2h 12h 24h 48h
1µM Tg
(aiii)
ATF4-LUC EXPRESSION (AU)
400000
300000
200000
100000
0
***
***
C 2h 12h 24h
1µM Tg
(bi)
1µM Tg
Time (h) - 2 12 24 48
p-IRE1α
p-JNK
JNK1/2
(bii)
p-IRE1α (AU)
25000
20000
15000
10000
5000
0
*
C 2h 12h 24h 48h
1µM Tg
(c)
1µM Tg
Time (h) - 2 12 24 48
Xbp1 (us)
Xbp1 (s)
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Xbp1 (s)
Xbp1 (us)

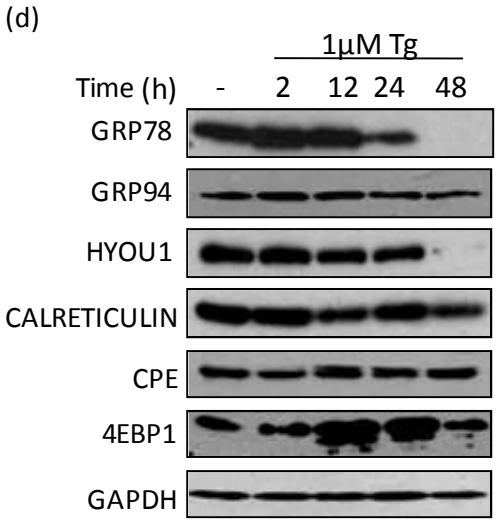


Figure 3.1. Thapsigargin induces ER stress in MIN6 cells. MIN6 cells grown in DMEM were treated with 1μM thapsigargin for the times indicated. (a) Cells were lysed and proteins resolved on SDS-PAGE and Western blotted using antisera against p-PERK, p-eIF2 and total eIF2α shown as a loading control. Nuclear extracts were western blotted using antisera against ATF4. (aii) Densitometric analysis of eIF2α phosphorylation is shown as a ratio of eIF2α. (aiii) ATF4 translation was assessed by measuring luciferase expression in cells infected with Ad-ATF4luc and treated with thapsigargin for the times indicated. (bi) Cells were lysed and proteins resolved on SDS-PAGE and Western blotted using antisera against p-IRE1, p-JNK and total JNK1/2 (bii) Densitometric analysis of IRE1α phosphorylation is shown in arbitrary units (c) Total RNA extracted using Trizol. After reverse transcription, XBP1 cDNA was amplified by PCR followed by incubation with Pst1 which cuts the PCR products produced from unspliced XBP1 into two smaller fragments of 290bp and 190bp. Spliced XBP1 (454bp) lacks the Pst1 restriction site and remains intact. (d) Cells were lysed and proteins resolved on SDS-PAGE and Western blotted using antisera against GRP78, GRP94, HYOU1, calreticulin, CPE, 4EBP1 and GAPDH. Western blots were scanned and quantified using SynGene GeneGnome System. Results shown are either representative or expressed as mean ± SEM of at least three independent experiments. *, $P<0.05$; ***, $P<0.001$ vs. control obtained using a one-way ANOVA followed by a Bonferroni post-test.

3.2.1.2 Temporal analysis of ER stress marker gene expression

To further characterise ER stress induction in MIN6 cells, the transcriptional expression profile of specific markers of ER stress and the activation of the UPR in response to thapsigargin was investigated using real-time quantitative PCR (RT-qPCR). Total RNA was extracted from MIN6 cells and cDNA was generated as outlined in Chapter 2. The resulting cDNA was amplified by RT-qPCR using gene specific oligonucleotide primers as listed in Chapter 2.

Thapsigargin induced a rapid (2 h) and significant increase in the mRNA expression levels of ATF4 and ATF3 (**Figure 3.2**), both genes known to be upregulated downstream of PERK activation. ATF4 expression peaked at 12 h, while ATF3 expression peaked at 2 h of thapsigargin treatment. Increases in ATF4 and ATF3 expression was followed by a steady decrease up to 48 h of thapsigargin exposure.

The expression of chaperone proteins and foldases under ER stress conditions is induced by the activation of the ATF6 and IRE1α arms of the UPR (Yamamoto, Yoshida et al. 2004). Thapsigargin induced a significant increase in the expression of calreticulin, HSPA5/BiP, HYOU1 and PDIA4 by 12 h, which was sustained for up to 24 h in all cases (**Figure 3.2**). These increases in mRNA expression did not however, reflect changes in protein expression (**Figure 3.1d**). The mRNA expression of UCP2, a ubiquitously expressed mitochondrial carrier which uncouples the respiratory chain from ATP synthesis was also assessed in this study (Zhang, Baffy et al. 2001). UCP2 has been suggested to inhibit glucose stimulated insulin secretion (GSIS) and its expression is reportedly upregulated under conditions of prolonged ER stress (Zhang, Baffy et al. 2001, Lameloise, Muzzin et al. 2001). Thapsigargin induced a rapid (2 h) and significant increase in UCP2 mRNA followed by a progressive decline back to basal levels by 48 h (**Figure 3.2**).

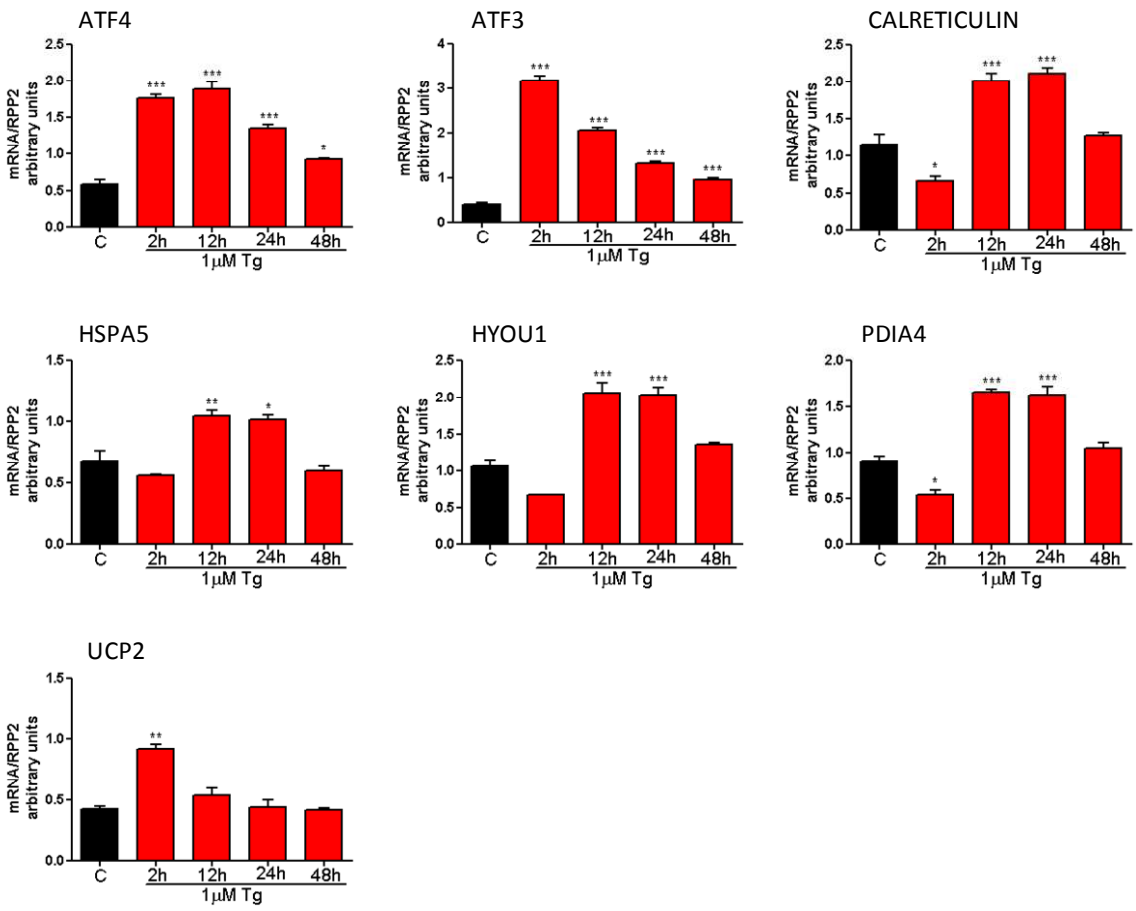


Figure 3.2. Temporal analysis of ER stress marker expression in thapsigargin induced ER stress. MIN6 cells grown in DMEM were treated with 1μM thapsigargin for indicated time points. Total RNA was extracted, cDNA generated and analysed by real-time qPCR. mRNA expression was normalised to the housekeeping gene, ribosomal protein P2 (RPP2). Results are mean ± SEM from three experiments. Statistical significance was determined using an unpaired two-tailed Student's t-test. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$ *vs.* control.

3.2.2 Chronic thapsigargin treatment induces apoptosis in MIN6 cells

While the induction of the UPR is an adaptive response to ER stress, chronic activation of this pathway has been shown to induce apoptosis in clonal β-cell lines (Cnop, Ladriere et al. 2007, Kharroubi, Ladriere et al. 2004). The trigger for the switch from an adaptive to an apoptotic response is currently unknown. To investigate whether thapsigargin induced ER stress results in the loss of cell viability and apoptosis, MIN6 cells were treated with thapsigargin over a 48 h time course and assessed for cell viability and activation of cleaved caspase-3 using the ApoLive Glo Multiplex assay (Promega). This assay was used to measure protease activity in live cells (viability) and caspase activity to determine the induction of apoptosis. The MTT assay which also measures metabolic activity (i.e. mitochondrial reductase enzyme activity), was used to measure cell viability.

A progressive decrease in cell viability was detected from 12 to 48 h of thapsigargin treatment in MIN6 cells measured using the MTT assay (**Figure 3.3ai**) and the ApoLive Glo assay (**Figure 3.3aii**). While ER stress signalling is detectable from 2 h of thapsigargin treatment (**Figure 3.1**), a significant loss of cell viability does not occur until 12 h of thapsigargin exposure. In addition to cell viability, the activity of the executor caspase-3 was also detected by Western blotting and the ApoLive Glo Multiplex assay. Cleavage of caspase-3 downstream of the caspase cascade is indicative of the activation of an apoptotic signalling machinery within the cell. Thapsigargin treatment induced a time-dependent increase in the expression of cleaved caspase-3 with significant increases observed after 24 and 48 h of thapsigargin exposure in comparison to untreated MIN6 cells (**Figure 3.3bi**). Caspase cleavage was also detected as early as 2 h after treatment with thapsigargin, peaked at 12 h and was sustained through the duration of the experiment (**Figure 3.3bii**).

The expression of CHOP downstream of PERK activation under ER stress conditions has been implicated in ER stress induced apoptosis. CHOP nuclear translocation, which is essential for the induction of CHOP induced apoptosis, is facilitated by its association with C/EBPβ (Chiribau, Gaccioli et al. 2010). The protein expression of CHOP in response to thapsigargin treatment was thus measured by Western blotting.

Thapsigargin induced a progressive increase in the expression of CHOP from 2 to 24 h of exposure in comparison to untreated cells (**Figure 3.3ci**). CHOP mRNA expression was also detected in response to thapsigargin treatment. In agreement with the protein expression data, thapsigargin induced a rapid (2 h) increase in the expression of CHOP, which peaked at 12 h followed by a decline up to 48 h (**Figure 3.3cii**). Thapsigargin also induced a rapid (2 h) increase in the expression of C/EBPβ followed by a progressive decline through the duration of the experiment (**Figure 3.3cii**).

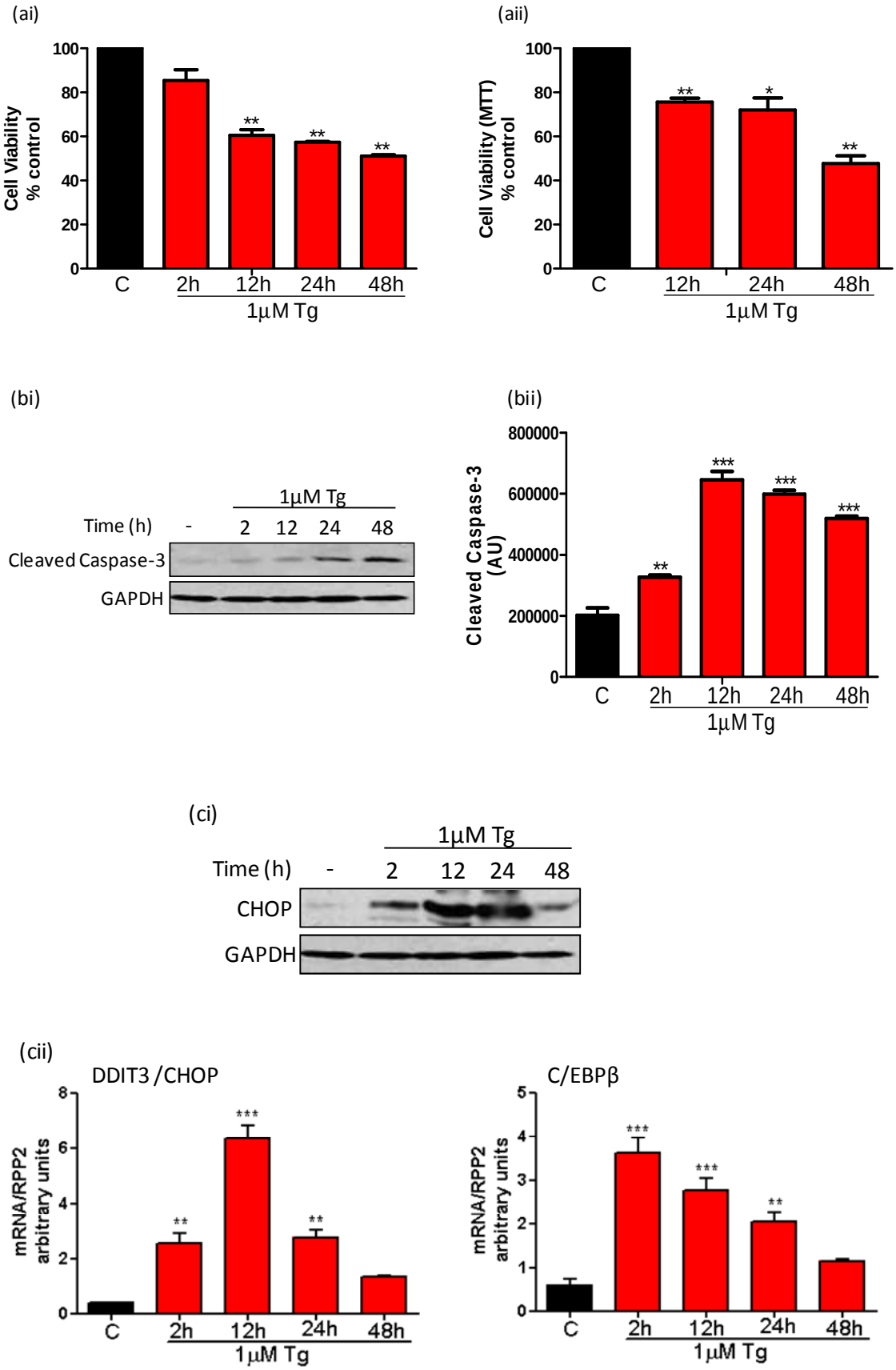


Figure 3.3. Chronic thapsigargin exposure induces apoptosis in MIN6 cells. MIN6 cells grown in DMEM were treated with 1μM thapsigargin for the time points indicated.

Experiments were stopped after time points lapsed and cell viability assessed by the (ai) ApoLive Glo assay (Promega) and (aii) MTT assay. (bi) Cells were lysed and proteins resolved on SDS-PAGE and Western blotted using antisera against cleaved caspase-3. GAPDH is shown as a loading control. (bii) Experiment was stopped after time course lapsed and cleaved caspase-3 activity was measured using the ApoLive Glo assay kit (Promega). (ci) Cells were lysed and nuclear extracts resolved on SDS-PAGE and Western blotted using antisera against CHOP. GAPDH is shown as a loading control. (cii) Total RNA was extracted and the expression of CHOP and C/EBPβ analysed by real-time qPCR. Expression was normalised to the housekeeping gene RPP2. Results shown are a mean ± SEM of at least three independent experiments. Statistical significance was determined using an unpaired two-tailed Students' t-test. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$ *vs.* control.

3.2.3 Palmitate and to a lesser extent, oleate induces ER stress signalling in MIN6 cells

3.2.3.1 Activation of the UPR

ER stress in response to fatty acid exposure has been reported both in clonal β-cell lines and primary β-cells (Cnop, Ladriere et al. 2007, Cunha, Hekerman et al. 2008, Laybutt, Preston et al. 2007). To investigate FFA induced ER stress signalling, MIN6 cells cultured in DMEM minus serum were treated with the unsaturated fatty acid, oleate, and the saturated fatty acid, palmitate, for up to 48 h and the expression of ER stress markers assessed by Western blotting. Control cells were treated with 0.67% BSA and a similar dilution of ethanol. The experiments in this report were carried out in the absence of serum as this experimental paradigm has been suggested to potentially mimic the conditions in type 2 diabetes (Maestre, Jordan et al. 2003). Serum deprivation, a widespread model for the treatment of cells with FFAs, has however been reported to be induce apoptosis independent of the treatment of cells (Maestre, Jordan et al. 2003). To overcome this potential problem, comparisons in the data have been made to control cells at each time point in order to eliminate the possible effects of serum deprivation on the results.

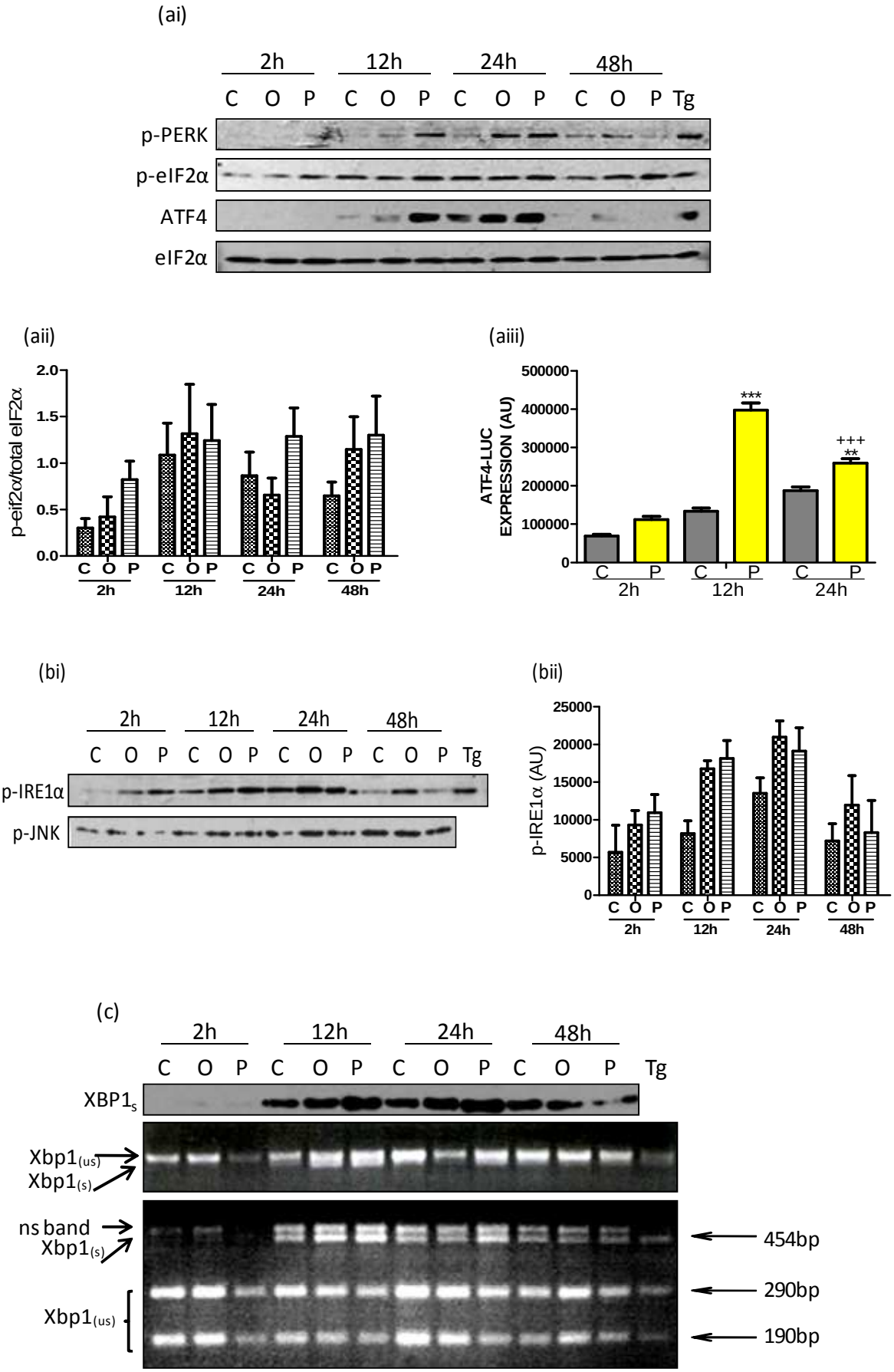
Palmitate treatment induced PERK phosphorylation which was apparent by 2 h of exposure and significant after 12 and 24 h (**Figure 3.4ai**). PERK phosphorylation in response to palmitate treatment however declined to basal levels after 48 h of chronic exposure (**Figure 3.4ai**). Oleate treatment also resulted in the phosphorylation of PERK albeit later (12 h) and to a lesser extent than palmitate (**Figure 3.4ai**). PERK phosphorylation in response to oleate treatment also peaked at 24 h and declined by 48 h of chronic exposure (**Figure 3.4ai**). These changes however did not parallel the phosphorylation of eIF2α as eIF2α phosphorylation was apparent from 2 h of palmitate treatment and was sustained for the duration of the experiment (**Figure 3.4ai, aii**). Palmitate treatment also resulted in a significant increase in ATF4 expression by 12 h which peaked at 24 h and declined significantly by 48 h of exposure (**Figure 3.4a**). Oleate also induced the expression of ATF4 by 12 h but to a lesser extent than palmitate. Oleate induced ATF4 expression also peaked at 24 h and declined

significantly by 48 h of exposure (**Figure 3.4a**). ATF4 translation in response to palmitate treatment was also assessed by measuring the expression of luciferase in cells infected with adenovirus expressing luciferase downstream of the 5' UTR of ATF4. Palmitate mediated the significant translational upregulation of luciferase after 12 h of treatment (**Figure 3.4aiii**). ATF4 expression remained significantly elevated after 24 h of exposure (**Figure 3.4aiii**).

Treatment of MIN6 cells with both FFAs also induced the phosphorylation of IRE1α from 2 h of exposure. Palmitate treatment induced the phosphorylation of IRE1α from 2 h which peaked at 24 h and declined significantly by 48 h (**Figure 3.4b**). Oleate treatment also resulted in the phosphorylation of IRE1α from 2 h albeit to a lesser extent than palmitate (**Figure 3.4b**). Oleate induced IRE1α phosphorylation peaked at 24 h and declined by 48 h but was found to be consistently higher than palmitate at the 48 h time point (**Figure 3.4b**). XBP1 splicing downstream of IRE1α activation was also assessed by Western blotting and PCR amplification. Palmitate induced significant XBP1 splicing by 12 h of treatment which peaked by 24 h and tapered off by 48 h (**Figure 3.4c**). Oleate induced a less pronounced splicing of XBP1 at 12 and 24 h and a decline to control levels by 48 h of exposure (**Figure 3.4c**). The phosphorylation of JNK in response to palmitate treatment was also assessed. In this study, neither oleate nor palmitate induced significant phosphorylation of JNK in comparison to control cells at all time points upon chronic exposure in MIN6 cells (**Figure 3.4b**).

Chaperone protein and foldase expression was also assessed in MIN6 cells treated with palmitate and oleate. No significant change was observed in the expression of the chaperone proteins GRP78, GRP94 and calreticulin in cells treated with either oleate or palmitate at all time points (**Figure 3.4d**). An increase in the expression of HYOU1 was observed at 2 and 12 h of palmitate treatment in comparison to untreated control cells (**Figure 3.4d**). CPE expression in response to FFA treatment was also assessed. CPE degradation has been suggested to precede the initiation of ER stress and β-cell death in response to palmitate treatment in MIN6 cells (Jeffrey, Alejandro et al. 2008). In my study however, both oleate and palmitate induced progressive CPE degradation from 12 h of FFA exposure up to 48 h in comparison to untreated control cells (**Figure 3.4d**). CPE degradation was observed to be more pronounced in palmitate treated

than in oleate treated MIN6 cells. Palmitate also induced a time dependent increase in the expression of 4EBP1 while oleate induced a milder response in comparison to palmitate (**Figure 3.4d**). The upregulation of 4EBP1 in response to both oleate and palmitate peaked at 24 h.


(ai)
2h
12h
24h
48h
C O P C O P C O P C O P Tg
p-PERK
p-eIF2α
ATF4
eIF2α
(aii)
p-eif2α/total eIF2α
2.0
1.5
1.0
0.5
0.0
C O P C O P C O P C O P
2h
12h
24h
48h
(aiii)
ATF4-LUC EXPRESSION (AU)
500000
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200000
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0
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C P C P C P
2h
12h
24h
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2h
12h
24h
48h
C O P C O P C O P C O P Tg
p-IRE1α
p-JNK
(bii)
p-IRE1α (AU)
25000
20000
15000
10000
5000
0
C O P C O P C O P C O P
2h
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24h
48h
(c)
2h
12h
24h
48h
C O P C O P C O P C O P Tg
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Xbp1(s)
Xbp1(us)
454bp
290bp
190bp

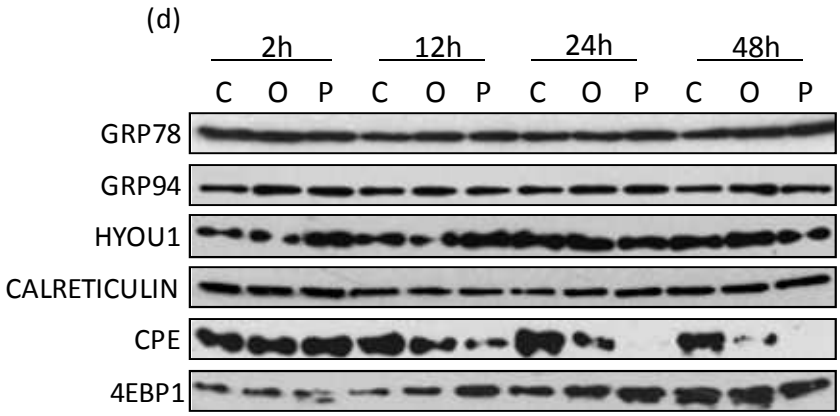


Figure 3.4. Palmitate, and to some extent oleate, induces ER stress in MIN6 cells. MIN6 cells grown in DMEM were treated with 0.5mM oleate or 0.5mM palmitate conjugated to 0.67% BSA or control 0.67% BSA with a similar dilution of ethanol for the time points indicated. (ai) Cells were lysed and proteins resolved on SDS-PAGE and Western blotted using antisera against p-PERK, p-eIF2 and total eIF2α shown as a loading control. Nuclear extracts were Western blotted using antisera against ATF4. (aii) Densitometric analysis of eIF2α phosphorylation is shown as a ratio of eIF2α. (aiii) ATF4 translation was assessed by measuring luciferase expression in cells pre-infected with Ad-ATF4luc for 24 h and treated with 0.5mM palmitate for the times indicated. Control cells were treated with a similar concentration of ethanol and 0.67% BSA. (bi) Cells were lysed and proteins resolved on SDS-PAGE and Western blotted using antisera against p-IRE1 and p-JNK. (bii) Densitometric analysis of IRE1α phosphorylation is shown in arbitrary units. (c) Total RNA extracted using Trizol. After reverse transcription, XBP1 cDNA was amplified by PCR followed by incubation with Pst1 which cuts the PCR products produced from unspliced XBP1 into two smaller fragments of 290bp and 190bp. Spliced XBP1 (454bp) lacks the Pst1 restriction site and remains intact. (d) Cells were lysed and proteins resolved on SDS-PAGE and Western blotted using antisera against GRP78, GRP94, HYOU1, calreticulin, CPE and 4EBP1. Western blots were scanned and quantified using SynGene GeneGnome System. Results shown are either representative or expressed as mean ± SEM of at least three independent experiments. *, $P<0.05$; ***, $P<0.001$ vs. control obtained using a one-way ANOVA followed by a Bonferroni post-test.

3.2.3.2 Temporal analysis of changes in the mRNA expression of ER stress markers in response to palmitate.

To further characterise palmitate induced ER stress in MIN6 cells, the transcriptional expression profile of specific markers of ER stress and the activation of the UPR was investigated using real-time quantitative PCR (RT-qPCR). MIN6 cells incubated in serum free media were treated with palmitate at different time points and control cells treated with a similar dilution of FA-free BSA and ethanol. Total RNA was extracted from the cells and cDNA generated as outlined in Chapter 2. The resulting cDNA was amplified by RT-qPCR using gene specific oligonucleotide primers as listed in Chapter 2.

Palmitate treatment induced a rapid (2 h) and significant increase in the expression of ATF4 and ATF3 mRNA in comparison to control cells at all time points assessed (**Figure 3.5**). Serum depletion however also appeared to induce a small but progressive increase in the expression of ATF4 in untreated control cells. The expression of chaperone proteins and foldases under ER stress conditions is induced by the activation of the ATF6 and IRE1α arms of the UPR. Palmitate had no effect on calreticulin mRNA expression and induced a rapid (2 h) increase in the expression of HSPA5/BiP which was however not sustained after 2 h (**Figure 3.5**). mRNA expression of the chaperone proteins HYOU1 and PDIA4 increased significantly at 12 h and 24 h of palmitate exposure respectively compared to BSA treated controls at the same time points. Serum depletion was observed to induce small but progressive increases in the mRNA expression of the chaperone proteins in the control cells. Palmitate did not induce the expression of UCP2 mRNA at any of the time points assessed (**Figure 3.5**). This is contrary to studies which show upregulation of UCP2 in INS-1 cells exposed to FFAs (Lameloise, Muzzin et al. 2001, Patanè, Anello et al. 2002).

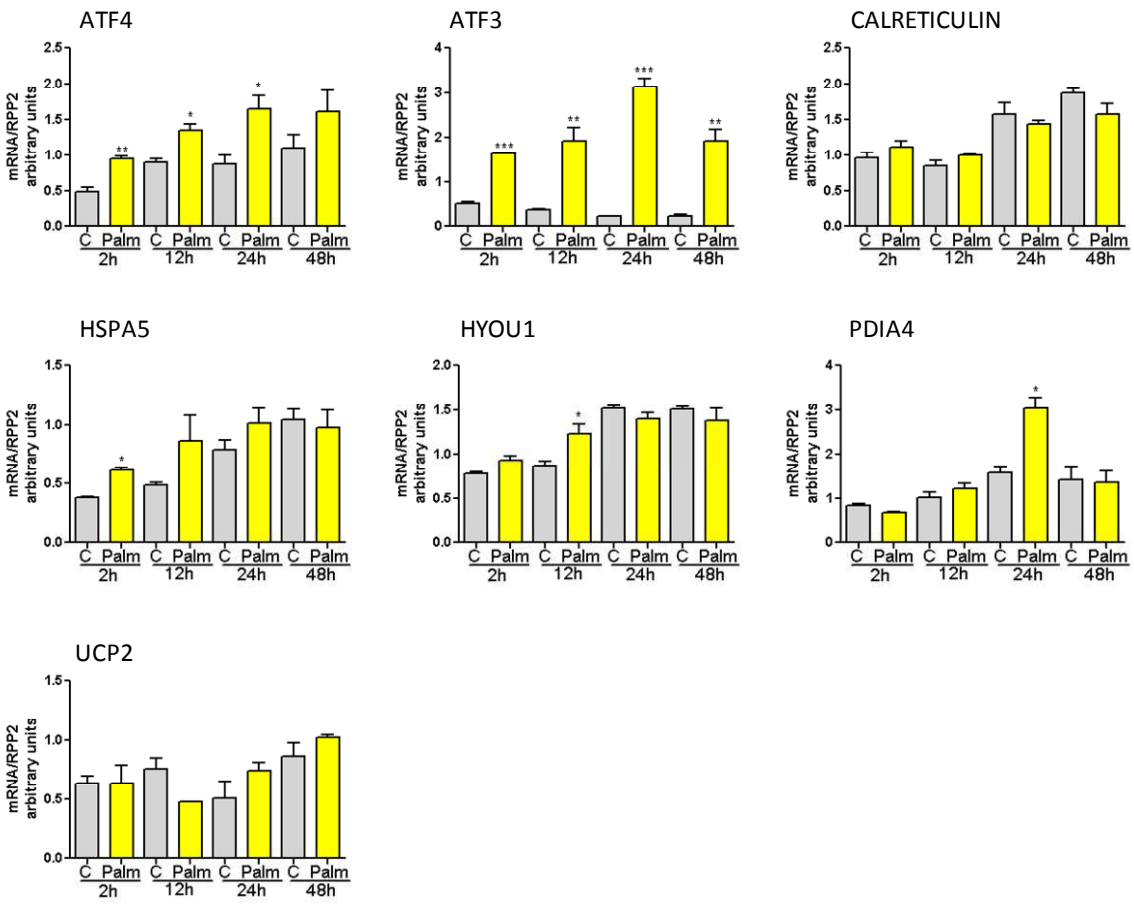


Figure 3.5. Temporal analysis of ER stress marker expression in palmitate induced ER stress. MIN6 cells grown in DMEM were treated with 0.5mM palmitate: 0.67% BSA for indicated time points. Control cells had a similar dilution of ethanol and 0.67% BSA only. RNA was extracted and analysed by quantitative real-time PCR. mRNA expression was normalised to the housekeeping gene, ribosomal protein P2 (RPP2). Results are mean ± SEM from three experiments. Statistical significance was determined using an unpaired two-tailed Students' t-test. *, *P*<0.05; **, *P*<0.01; ***, *P*<0.001 *vs.* control.

3.2.4 Chronic palmitate exposure induces apoptosis in MIN6 cells

Chronic exposure to elevated levels of FFAs has been shown to induce apoptosis in human pancreatic β-cells in vitro and in β-cell lines (Laybutt et al. 2007; Lupi et al. 2002, Lai et al. 2008). To characterise the effect of FFAs on MIN6 viability, MIN6 cells were treated with palmitate or oleate in the absence of serum over a 48 h time course and assessed for viability and markers of apoptotic signalling. Changes in MIN6 cell morphology in response to palmitate treatment were initially assessed using light microscopy. By 24 h of palmitate exposure, some cells were observed to round up and detach from the plate, indicative of cell death (**Figure 3.6a**). By 48 h of palmitate treatment, significant detachment and rounding up of cells was observed with a clear variation in cell morphology, indicative of significant stress and apoptosis in palmitate treated cells (**Figure 3.6a**).

The viability of MIN6 cells treated with oleate or palmitate over 48 h was also assessed using the MTT assay. While palmitate induced significant loss of cell viability over time, oleate treatment had no observed effect on MIN6 cell viability (**Figure 3.6b**). To investigate whether palmitate induced a loss of cell viability through the induction of apoptosis, the expression of cleaved caspase 3 was also assessed. Palmitate induced a progressive increase in the expression of cleaved caspase-3 from 12 to 48 h of exposure in comparison to control cells (**Figure 3.6c**). By comparison, oleate treatment had no significant effect on the expression of cleaved caspase-3 in MIN6 cells at all time points assessed (**Figure 3.6c**). This indicates that palmitate but not oleate induces cell death through the apoptotic pathway in MIN6 cells. Laybutt et al., also reported significant induction of cleaved caspase-3 expression in MIN6 cells treated with palmitate for 48 h (Laybutt et al. 2007).

To investigate whether CHOP expression is increased in response to FFAs, MIN6 cells were treated with either oleate or palmitate for up to 48 h and changes in CHOP expression were assessed by Western blotting. In this study, both oleate and palmitate induced the expression of CHOP protein by 12 h of FA treatment to levels which remained elevated for up to 24 h. However by 48 h, the expression of CHOP had returned to almost basal levels in both palmitate and oleate treated cells (**Figure 3.6di**). Changes in CHOP mRNA expression in response to palmitate exposure were

also assessed by RT-qPCR. Palmitate induced a significant increase in the mRNA expression of CHOP by 12 h compared to its control (**Figure 3.6dii**). This increase was sustained throughout the course of the experiment (**Figure 3.6dii**). Increased expression of CHOP mRNA and protein in response to palmitate treatment has been reported both in MIN6 and INS-1 cell lines (Cunha, Hekerman et al. 2008; Laybutt, Preston et al. 2007; Lai, Bikopoulos et al. 2008). Oleate induced CHOP expression has also been reported in INS-1E cells albeit to a lesser extent than palmitate (Cnop, Ladriere et al. 2007, Kharroubi, Ladriere et al. 2004). The pro-apoptotic function of CHOP has been reported to be mediated by an interaction with its dimerisation partner C/EBPβ which facilitates translocation into the nucleus (Ron, Habener 1992). In correlation with CHOP expression, C/EBPβ mRNA expression was also significantly upregulated from 2 h of palmitate treatment and sustained for the duration of the experiment (**Figure 3.6dii**). There was however, no significant change in the expression of C/EBPβ at the level of protein expression (**Figure 3.6di**).

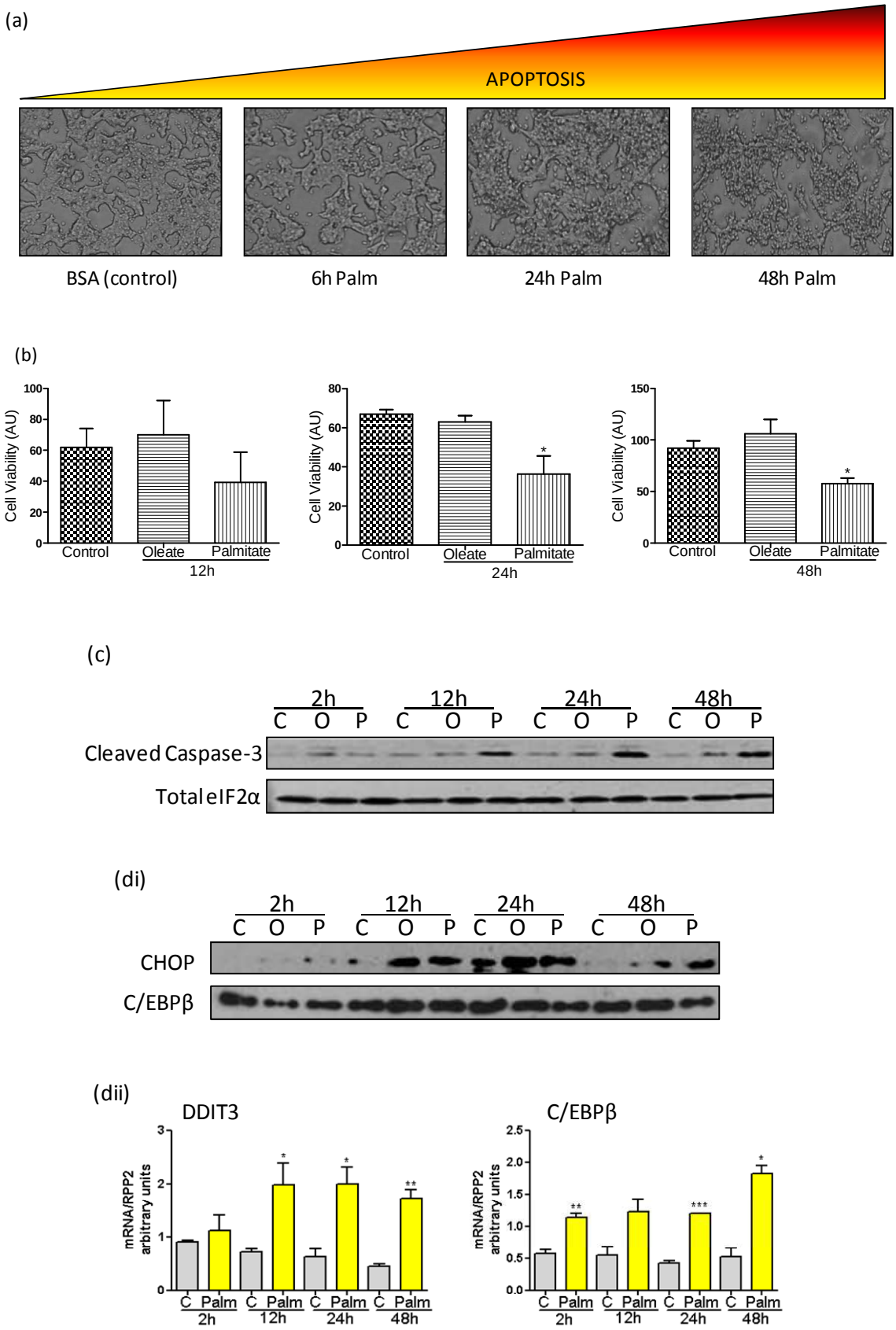


Figure 3.6. Chronic palmitate exposure induces apoptosis in MIN6 cells. MIN6 cells grown in DMEM were treated with 0.5mM oleate or 0.5mM palmitate conjugated to

0.67% BSA or control 0.67% BSA with a similar dilution of ethanol for the time points indicated. (a) Morphological in MIN6 cells chronically exposed to 0.5mM palmitate at indicated time points was detected using light microscopy. (b) Experiments were stopped after time points lapsed and cell viability assessed by MTT assay (c) Cells were lysed and proteins resolved on SDS-PAGE and Western blotted using antisera against cleaved caspase-3. Total eIF2α is shown as a protein loading control. (di) Cells were lysed and nuclear extracts were Western blotted using antisera against CHOP (cii) Total RNA was extracted and the expression of CHOP and C/EBPβ analysed by real-time qPCR. Expression was normalised to the housekeeping gene RPP2. Results shown are either representative or expressed as mean ± SEM of at least three independent experiments. Statistical significance was determined using an unpaired two-tailed Students' t-test. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$ *vs.* control.

3.3 Discussion

Using the MIN6 clonal β-cell line, data presented in this chapter shows that ER stress is inducible in this cell line by a known ER stress inducer thapsigargin. I have also shown that both oleate and palmitate may induce ER stress in our MIN6 cells albeit with different propensity. Despite the induction of ER stress by both fatty acids, only palmitate induced apoptosis in the MIN6 cells, indicative of differential signalling by unsaturated and saturated fatty acids.

In order to initially characterise the induction of ER stress in our MIN6 cell line, cells were treated with a known inducer of ER stress, thapsigargin. Thapsigargin induces ER stress through the inhibition of SERCA pumps on the ER membrane resulting in the irreversible depletion of ER Ca^{2+} levels (Lytton, Westlin et al. 1991). Consistent with the irreversible effects of thapsigargin on the SERCA pumps, chronic phosphorylation of PERK was observed in the MIN6 cells for the duration of the experiment. Despite the sustained activation of PERK, eIF2α phosphorylation levels diminished progressively after 12 h of thapsigargin exposure (**Figure 3.1**). Although the expression of GADD34 was not assessed in this study, this decrease in eIF2α phosphorylation state is indicative of the activation of a negative feedback mechanism downstream of PERK. Increases in the expression of ATF4 and CHOP protein and mRNA were also observed, events which occur downstream of PERK activation. This transient upregulation was however followed by a progressive diminution in both mRNA and protein levels. The short lived upregulation of these ER stress markers may be attributed to the inherent instability of ATF4 and CHOP mRNA and protein (Rutkowski, Arnold et al. 2006) even in the presence of persisting ER stress signals such as thapsigargin treatment.

The activation of the IRE1 signalling pathway in response to thapsigargin treatment was also assessed. Despite the irreversible depletion of ER Ca^{2+} and thus, the chronic induction of ER stress by thapsigargin, IRE1 phosphorylation was observed to be time dependent (**Figure 3.1**). Thapsigargin induced IRE1 phosphorylation peaked at 12 h and was observed to decline after 24 h. In addition, XBP1 splicing was observed to peak at 12 and 24 h, with diminution to almost basal levels observed at 48 h. These

data correlate with similar observations made in HEK293 cells (Lin, Li et al. 2007). The attenuation of IRE1 signalling in the face of persisting ER stress is particularly interesting as the activity of this transducer protein has been suggested to enhance cell survival. In the study by Lin et al., experimental sustenance of IRE1 activity was found to enhance cell survival, thereby demonstrating a link between IRE1 activity and cell survival (Lin, Li et al. 2007). The attenuation of IRE1 activity in the MIN6 cells despite the presence of an ER stressor therefore suggests a switch off of protective IRE1 signalling in the cells. Despite the attenuation of IRE1 activity, the phosphorylation of JNK was sustained in the MIN6 cells. This may have occurred as a result of the amplification of signals downstream of the IRE1 which perhaps, do not require sustained activation of the proximal sensor for their propagation.

Despite the evident activation of PERK and IRE1 in response to thapsigargin treatment, no robust induction of chaperone proteins was observed at the protein level. Indeed, while chaperone protein mRNA expression was significantly induced and sustained up to 24 h, this did not reflect in the expression of proteins. A small increase in BiP protein expression was observed at 2 and 12 h, followed by a progressive diminution. It is unclear why no increase in chaperone protein expression was observed even in the presence of increased mRNA and the apparent release of translation inhibition by the observed dephosphorylation of eIF2α over time. It may be proffered however, that a residual pool of phosphorylated eIF2α, which may occur as a result of the chronic activation of PERK in response to thapsigargin treatment, is sufficient to sustain the inhibition of protein translation despite the time dependent dephosphorylation of eIF2α observed in this study. In addition, activated IRE1 has been reported to degrade ER-targeted mRNAs under ER stress conditions (Hollien, Weissman 2006). The upregulated mRNAs may thus be degraded by this mechanism thereby inhibiting their translation. This would result in the inhibition of chaperone protein expression despite upregulation at the level of transcription.

As expected, the chronic treatment of MIN6 cells with thapsigargin resulted in significant loss of cell viability and the time dependent expression of cleaved caspase-3, an indicator of irreversible apoptotic induction. The depletion of Ca^{2+} from the ER results in an increase in cytoplasmic Ca^{2+}, which may result in the induction of

apoptosis through several signalling events including the release of *cytochrome c* from the mitochondria resulting in apoptosome formation, and the activation of calpains, which regulate caspase activity. In addition, chronic ER Ca^{2+} depletion induces ER stress and as a consequence, the expression of pro-apoptotic CHOP downstream of PERK activation. Indeed, CHOP was expressed in the MIN6 cells in response to thapsigargin treatment, although its expression was observed to diminish over time as discussed above.

In addition to thapsigargin, the induction of ER stress signalling in response to FFAs was also characterised in our MIN6 cells. Chronic elevation in circulating plasma FFAs have been suggested to contribute to the development of type 2 diabetes through the induction of ER stress in the β-cells and consequently, β-cell apoptosis. The expression of ER stress markers has previously been reported in pancreas sections and islets isolated from animal models of obesity and diabetes and human subjects (Laybutt, Preston et al. 2007, Huang, Lin et al. 2007). In addition, chronic exposure of β-cell lines to FFAs, in particular saturated fatty acids such as palmitate, has been shown to induce ER stress (Cunha, Hekerman et al. 2008, Karaskov, Scott et al. 2006, Lai, Bikopoulos et al. 2008). The differential effects of oleate and palmitate have been widely reported. Unsaturated fatty acids such as oleate and palmitoleate have been reported to be potently cytoprotective, and can attenuate the loss of cell viability induced by saturated fatty acids (Welters, Tadayyon et al. 2004, Diakogiannaki, Dhayal et al. 2007). Although oleate has been shown in many instances to be cytoprotective or to exert no detrimental effects on clonal β-cell lines (Laybutt, Preston et al. 2007, Diakogiannaki, Dhayal et al. 2007), some reports show evidence of apoptosis in response to oleate treatment (Kharroubi, Ladriere et al. 2004, Karaskov, Scott et al. 2006). In general however, there is a greater propensity for palmitate to induce ER stress and apoptosis in clonal β-cell lines and islets in comparison to oleate.

Data in this study show that both oleate and palmitate induced the phosphorylation of PERK and eIF2α in response to acute (2-12 h) and chronic (24-48 h) exposure. Palmitate mediated PERK and eIF2α phosphorylation however occurred more rapidly and more markedly in comparison to oleate. A similar observation was made in a study by Cunha et al., which reports the marked activation of PERK and IRE1 pathway by

palmitate and much less so by oleate in INS-1E cells (Cunha, Hekerman et al. 2008). PERK activation was also evidenced by the expression of ATF4 and CHOP in palmitate and oleate treated cells. In keeping with previous published observations, palmitate induced ATF4 and CHOP expression occurred more rapidly and was more pronounced than oleate induced changes. The induction of CHOP by oleate, although to a lesser extent than palmitate, was unexpected as oleate is generally proposed to exert cytoprotective effects. It was even more surprising then to find that despite the upregulation of pro-apoptotic CHOP expression by both FFAs, only palmitate was observed to induce apoptosis in the MIN6 cells. This is in contrast to the observations made by Cunha et al., where although both palmitate and oleate induced CHOP expression, albeit to markedly lower extents by oleate, both FFAs also induced apoptosis in INS-1E cells (Cunha, Hekerman et al. 2008). This difference may be due to the susceptibility of INS-1E cells to ER stress and apoptosis, as MIN6 cells are reported to be more robust and to possess more resistance to ER stress than INS-1E cells (Lai, Bikopoulos et al. 2008). In another report, only palmitate was found to induce the expression of CHOP and apoptosis whilst oleate had no effect on CHOP expression in MIN6 cells (Laybutt, Preston et al. 2007). While their observation of palmitate induced apoptosis was in correlation with my study, they did not observe oleate induced CHOP expression. This difference may have occurred as Laybutt et al., carried out their experiments in the presence of serum, conditions which have been shown to induce milder ER stress and require prolonged incubation time to observe cytotoxic effects in normal β-cells (Karaskov, Scott et al. 2006). Despite the differences in comparison to other studies, the observation in this study that both oleate and palmitate induced ER stress and CHOP expression, but only palmitate induced apoptosis in our MIN6 cells, suggests that ER stress is not a major mechanism through which FFAs induce apoptosis in β-cells. This hypothesis is supported by the observation in a study by Lai et al., which found that palmitate was able to induce apoptosis in INS-1 cells even at concentrations which induced minimal or no ER stress (Lai, Bikopoulos et al. 2008).

In addition to PERK signalling, both oleate and palmitate were observed to induce the phosphorylation and thus, activation of IRE1 in our MIN6 cells. Similar to the kinetics of IRE1 activation observed in thapsigargin treated cells, IRE1 phosphorylation peaked

at 24 h, followed by a diminution by 48 h. It was however, consistently observed that although the levels diminished over time, IRE1 phosphorylation at 48 h was higher in oleate treated cells in comparison to palmitate treated cells (**Figure 3.4b**). The levels of spliced XBP1 were also observed to mirror the changes observed in IRE1 activity. This is an interesting observation as the state of IRE1 phosphorylation and thus, activation has been suggested to mediate cell survival at least in HEK293 cells (Lin, Li et al. 2009). The sustained phosphorylation of IRE1 in oleate treated cells may therefore play a role in the sustenance of cell viability as observed in my study. Caution must however be taken in the interpretation of this data as overexpression of the spliced form of XBP1 was reported to induce apoptosis in a study in rat islets and FACS-purified β-cells (Allagnat, Christulia et al. 2010). This suggests that while sustained IRE1 activity may mediate cell survival, the mechanisms through which this action occurs may be independent of XBP1 splicing. While no reports have shown mechanisms through which IRE1 may mediate cell survival in β-cells, its activity has been reported to be necessary for differentiation in plasma cell biogenesis (Reimold, Iwakoshi et al. 2001, Zhang, Wong et al. 2005) and liver development (Reimold, Etkin et al. 2000). Studies into the possible mechanisms through which IRE1 signalling may mediate cell survival in β-cells may therefore prove useful in determining the mechanisms through which ER stress induced apoptotic signals are propagated. Although the potential role for IRE1 mediated cell survival is unexplored, the chronic activation of IRE1 signalling has been implicated in the induction of apoptosis through the phosphorylation and subsequent activation of JNK (Urano, Wang et al. 2000). While others have reported an increase in JNK activity in palmitate treated INS-1E cells (Cunha, Hekerman et al. 2008), oleate or palmitate exposure did not induce JNK phosphorylation in our MIN6 cells (**Figure 3.4bi**). This suggests, at least in our MIN6 cells, that IRE1 mediated JNK phosphorylation is not a mechanism through which apoptosis was induced.

Similar to the observations made in thapsigargin treated cells, activation of PERK and IRE1 by the FFAs did not induce a robust expression of chaperone proteins as was expected. It was particularly surprising that the major ER chaperone protein BiP/GRP78, was not induced in response to FFA treatment as the IRE1 signalling pathway has been described to be involved in the transcription of ER chaperone genes

such as BiP (Yoshida, Matsui et al. 2001). In addition, the ATF6 pathway, which was not assessed in this study, has also been shown to induce BiP transcription under ER stress conditions (Harding, Calfon et al. 2002, Li, Baumeister et al. 2000). The lack of induction of BiP and other chaperone proteins suggests that perhaps, the ATF6 pathway was not activated in our MIN6 cells in response to FFA exposure. It also suggests that the beneficial aspects of the UPR induction were bypassed in the MIN6 cells in response to both saturated and unsaturated fatty acid exposure.

The question of how FFAs induce ER stress and how palmitate in particular induces apoptosis in β-cells remains unsolved. While possible mechanisms have been outlined in the introduction to this chapter, the exact mechanisms and pathways are unknown. It is clear however, that while both oleate and palmitate are capable of stimulating the activation of the UPR in the MIN6 clonal β-cell line, unidentified signalling events specific to palmitate may be activated resulting in palmitate specific induction of apoptosis.

3.4 Conclusion

The data presented in this chapter confirm that the induction of chronic ER stress can stimulate the activation of apoptosis in thapsigargin treated MIN6 cells. I have also shown that both the unsaturated fatty acid oleate, and the saturated fatty acid palmitate, can induce ER stress in our MIN6 cells albeit more markedly by palmitate. It was however observed that there was no direct correlation between the induction of UPR signalling and apoptosis. This suggests that palmitate induced apoptosis in β-cells, at least *in vitro*, occurs via an alternative mechanism to ER stress. In order to rule out the involvement of fatty acid induced ER stress in β-cell apoptosis, further experimentation is required to determine alternative mechanisms specific to palmitate, which may be responsible for the induction of apoptosis in β-cells.

CHAPTER 4

Chapter 4: Determination of the role of eIF2α phosphorylation on ER-stress evoked pancreatic β-cell death.

4.1 Introduction

The transduction of ER stress signals in cells is mediated by the activity of ER-transmembrane proteins such as PERK, which has also been shown to regulate fundamental physiological and developmental functions. In humans, loss of function mutations in PERK have been shown to cause a rare form of permanent neonatal diabetes called Walcott Rallison Syndrome (WRS) characterised by neonatal diabetes as a result of β-cell loss, exocrine pancreas atrophy, and growth retardation (Delepine, Nicolino et al. 2000). PERK knockout mice have also been reported to exhibit similar defects as humans with WRS (Harding, Zeng et al. 2001, Zhang, McGrath et al. 2002) thereby providing further evidence for the requirement for PERK in β-cell survival. These effects of loss of function mutations in PERK or PERK deficiency have been suggested to be mediated through the phosphorylation state of eIF2α, as non-phosphorylatable eIF2α Ser 51 to Ala knock-in (eIF2Ser51Ala) embryos and neonates exhibited a deficiency in pancreatic β-cells and died within 18 h of birth (Scheuner, Song et al. 2001). Together these studies provide evidence that PERK-dependent eIF2 phosphorylation is essential for β-cell survival (Scheuner, Song et al. 2001, Harding, Zhang et al. 2000). Yet β-cell specific conditional PERK knockout in adult mice provided evidence that PERK is not essential for the maintenance of β-cell function and glucose homeostasis (Zhang, Feng et al. 2006). These animals are however, likely to retain residual PERK activity/expression. Heterozygous eIF2α eIF2Ser51Ala knock-in mice survive but when fed on a HFD, develop diabetes due to β-cell failure (Scheuner, Mierde et al. 2005). There is evidence to suggest that the inability to phosphorylate eIF2α in β-cells results in increased protein synthesis and the accumulation of unfolded/misfolded proteins in the ER which may eventually induce cell death as the accumulation of dense material in the ER and distorted ER morphology was observed by electron microscopy in $PERK^{-/-}$ islets (Harding, Zeng et al. 2001).

While the aforementioned studies suggest that impaired PERK signalling is detrimental to cell survival, chronic PERK phosphorylation in response to ER stress inducers such as thapsigargin, or more physiological ER stress inducers such as palmitate, have also been shown to induce cell death in both MIN6 and INS-1E β-cell lines (Kharroubi, Ladriere et al. 2004, Cunha, Hekerman et al. 2008, Laybutt, Preston et al. 2007). A study in which chronic eIF2α phosphorylation was induced using a selective inhibitor of eIF2α dephosphorylation salubrinal, reported that chronic eIF2α phosphorylation potentiated the apoptotic effect of ER stress inducers such as palmitate in INS-1E cells (Cnop, Ladriere et al. 2007). This observation suggests that excessive eIF2α phosphorylation is poorly tolerated in β cells and may induce apoptotic signalling.

In addition to reducing cell survival, loss of function mutations in PERK have also been suggested to induce an increase in the activity of other ER stress pathways. PERK knockout was reported to result in the increased phosphorylation of IRE1α and cleavage of pro-apoptotic caspase-12 in mouse embryonic stem cells (Harding, Zhang et al. 2000). Increased phosphorylation of IRE1α was also observed in the pancreas of PERK knockout mice in comparison to wild type mice (Harding, Zeng et al. 2001). It is suggested that the compensatory activation of IRE1α may contribute to the induction of apoptosis as IRE1α overexpression was identified as a potent inducer of cell death in Chinese hamster ovary (CHO) cells (Wang, Harding et al. 1998). Active IRE1α may also induce apoptosis through its ability to activate JNK thereby coupling ER stress to apoptotic signals (Urano, Wang et al. 2000). Indeed, the overexpression of spliced XBP-1, the product of ER stress induced IRE1 activation, was reported to induce apoptosis in dispersed rat islets and FACS-purified β-cells (Allagnat, Christulia et al. 2010). The IRE1 pathway may therefore also play a significant role in the modulation of apoptotic signals in disease states such as diabetes. Alternatively, the chronic activation of PERK signalling may induce apoptosis through the upregulation of ATF4 expression and its downstream targets such as the pro-apoptotic transcription factor known as C/EBP homologous protein (CHOP) (Harding, Novoa et al. 2000b). CHOP, a member of the CCAAT/enhancer binding protein (C/EBP) family is also known as growth-arrest and DNA-damage inducible gene 153 (GADD153/DDIT3) (Fornace, Nebert et al. 1989). The transcription factor is however induced by ER stress more than growth arrest or DNA

damage (Wang, Lawson et al. 1996). CHOP has been identified as one of the most highly upregulated genes under conditions of chronic ER stress activity (Harding, Novoa et al. 2000b, Okada, Yoshida et al. 2002), and has been shown to induce the downstream expression of another growth-arrest and DNA-damage inducible gene 34 (GADD34) (Marciniak, Yun et al. 2004). GADD34 promotes the dephosphorylation of eIF2α by forming a complex with and targeting the catalytic subunit of the protein phosphatase 1 (PP1c) to the phosphorylation site of eIF2α (Novoa, Zeng et al. 2001, Brush, Weiser et al. 2003). The expression of pro-apoptotic CHOP under conditions of chronic ER stress signalling is identified as a marker of apoptosis. The downstream expression of GADD34 however, appears to play a dual role in the propagation of both survival and apoptotic signals. GADD34 induction was shown to be necessary for the survival of ER stress in cultured NIH-3T3 cells by facilitating the synthesis of UPR target proteins such as GRP78 and GRP94 (Novoa, Zhang et al. 2003). It has however also been suggested that GADD34 mediated recovery of protein synthesis promotes ER stress conditions by increasing protein load within the lumen of the ER. Indeed, MEF cells expressing a mutated form of GADD34 were partially protected against ER stress induced apoptosis in comparison to controls (Marciniak, Yun et al. 2004).

4.1.1 Aims

The aim of this study was to determine the role of PERK-dependent eIF2α phosphorylation in ER stress induced β-cell death.

4.2 Results

4.2.1 Adenovirus expressing an N-terminal truncation mutant of GADD34 inhibits eIF2α phosphorylation.

To investigate the role of the PERK signalling pathway in ER stress induced cell death, adenovirus expressing an N-terminal truncation mutant of GADD34 was generated as previously described (Gomez, Powell et al. 2008) and purified by Dr E Gomez following the manufacturer's instructions in the AdEasy Vector System kit (Qbiogene). This mutant form of GADD34 constitutively directs protein phosphatase 1 to the alpha subunit of eIF2α thereby inducing chronic dephosphorylation of the protein under ER stress conditions. To determine its infectivity and functionality, MIN6 cells were infected with increasing concentrations of the virus for 48 h prior to treatment with 1μM thapsigargin for 2 h. Increasing concentrations of AdGADD34ΔN induced progressive dephosphorylation of eIF2α in the presence of thapsigargin with maximal dephosphorylation observed in MIN6 cells infected with 10μl/ml of the virus (**Figure 4.1**). Under this condition, >90% of the cells were infected as determined by the detection of enhanced green fluorescent protein (EGFP) expression using fluorescence microscopy. As controls, MIN6 cells were also infected with purified recombinant adenovirus containing EGFP only (AdEmpty-EGFP). The expression of EGFP alone had no effect on the phosphorylation state of eIF2α in response to thapsigargin treatment (**Figure 4.1**). The expression of EGFP in cells infected with AdEmpty-EGFP and AdGADD34ΔN were compared by Western blotting in order to determine virus concentrations required for further experimentation as a measure of EGFP expression. 10μl of AdGADD34ΔN and 5μl of AdEmpty per ml was used for subsequent experiments in this study.

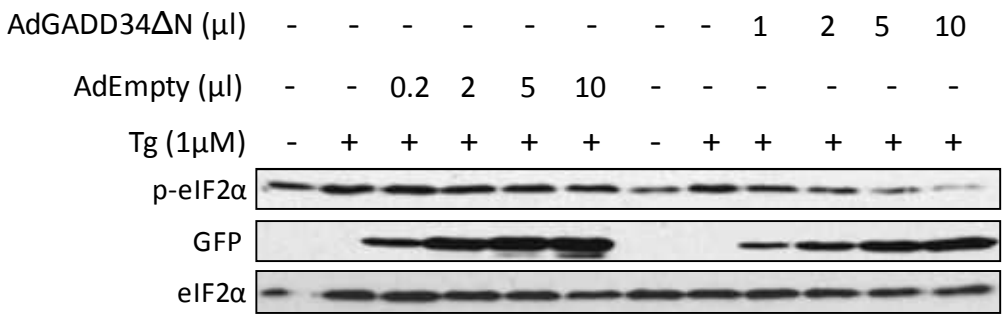


Figure 4.1. MIN6 cells grown in DMEM were either mock infected, infected with increasing volumes of Ad-Empty virus (AdEmpty) or AdGADD34ΔN for 48 h. Following infection, the cells were treated with 1µM thapsigargin for 2 h. Cells were lysed and proteins resolved on SDS-PAGE and Western blotted using antisera against phosphorylated eIF2α, GFP and total eIF2α as a loading control. n=1.

4.2.2 Inhibition of eIF2α phosphorylation has no effect on the downstream expression of ATF4 and CHOP in thapsigargin treated MIN6 cells.

To determine the effect of forced eIF2α dephosphorylation on the expression of downstream targets of the PERK pathway in response to ER stress, MIN6 and INS-1E cells infected with AdEmpty and AdGADD34ΔN were treated with 1μM thapsigargin for 24 h. Changes in the expression and phosphorylation status of downstream targets of the PERK signalling pathway were then determined by Western blotting. Thapsigargin treatment of both mock and AdEmpty infected MIN6 and INS-1E cells led to an increase in eIF2α phosphorylation and in the expression of ATF4 and CHOP (**Figure 4.2a, b**). In cells expressing GADD34ΔN, the inhibition of eIF2α phosphorylation was observed in control and thapsigargin treated (**Figure 4.2a, b**) but surprisingly had no effect on the downstream expression of ATF4 and CHOP (**Figure 4.2ai, aii, b**). Indeed in MIN6 cells (**Figure 4.2ai,aii**) and more prominently in INS-1E cells (**Figure 4.2bi**), the inhibition of eIF2α phosphorylation in control cells appeared to induce the expression of ATF4 and CHOP in control cells. The data from these experiments suggest that the ER stress induced expression of ATF4 and CHOP in MIN6 and INS-1E is mediated by a mechanism that is independent of eIF2α phosphorylation.

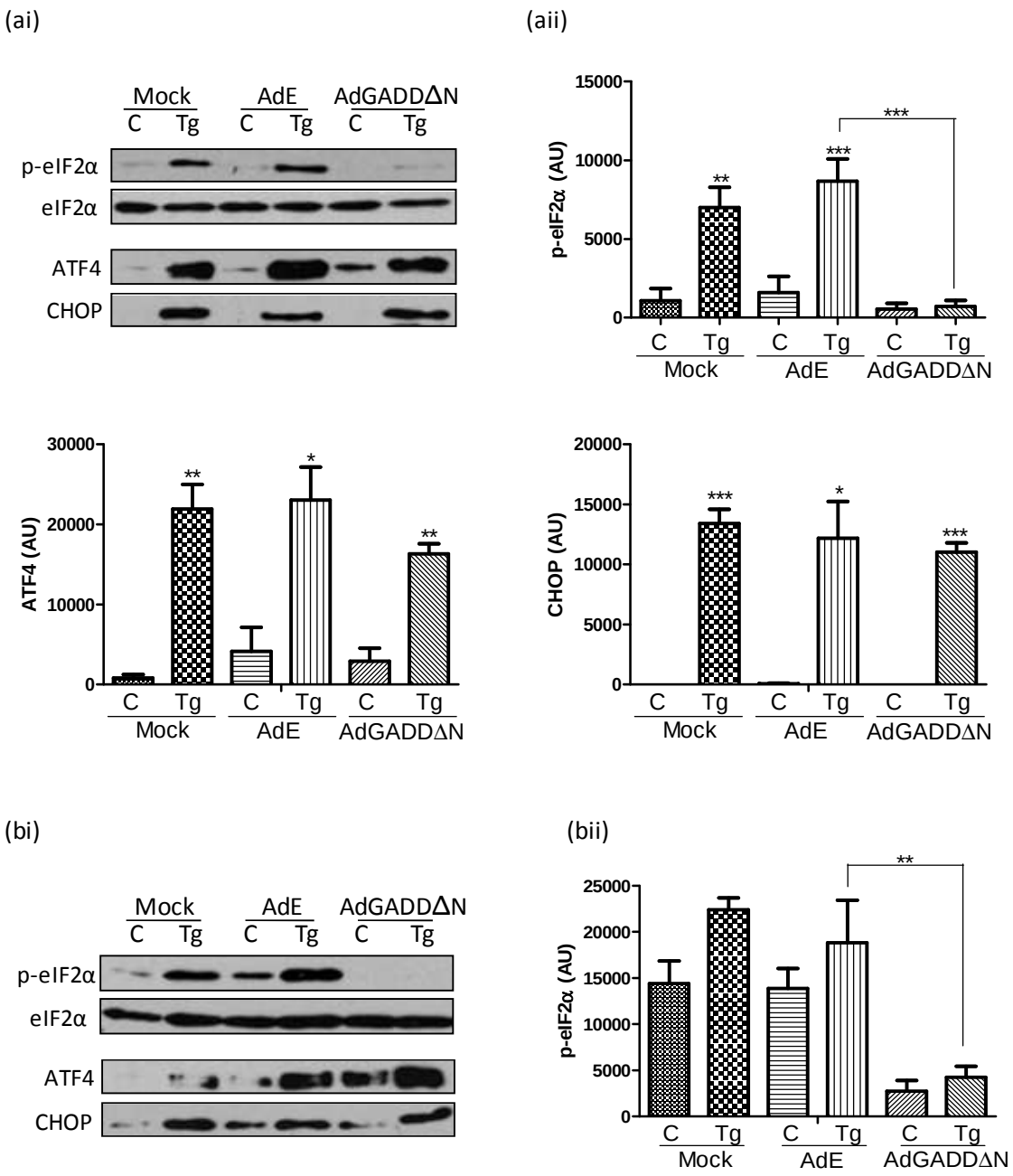


Figure 4.2. Inhibition of thapsigargin induced eIF2α phosphorylation by AdGADD34ΔN does not inhibit the downstream expression of ATF4 and CHOP. (a) MIN6 and (b) INS-1E cells were mock infected, infected with AdEmpty (AdE) or AdGADD34ΔN (AdGADDΔN) for 24 h. Following infection, cells were treated with 1μM thapsigargin for a further 24 h. Cells were lysed and proteins resolved on SDS-PAGE and Western blotted using antisera against phosphorylated eIF2α and total eIF2α as a loading control. Nuclear extracts were resolved on SDS-PAGE and Western blotted using antisera against ATF4 and CHOP. Densitometric analysis of (aii) eIF2α phosphorylation, ATF4 and CHOP expression in MIN6 cells and (bii) eIF2α phosphorylation in INS-1E cells is expressed in arbitrary units. Results shown are either representative or expressed as mean ± SEM of at least three independent experiments. Experiment for nuclear

extract in INS-1E cells n=1. Statistical analysis was carried out using an unpaired two-tailed Students' t-test. *, P<0.1; **, P<0.01; ***, P<0.001 *vs* control.

4.2.3 Inhibition of eIF2α phosphorylation induces cleaved caspase-3 expression and apoptosis in thapsigargin treated MIN6 cells.

To determine the role of the PERK signalling pathway in ER stress induced β-cell death, MIN6 cells infected with AdGADD34ΔN were treated with thapsigargin and the expression of cleaved caspase-3, an indicator of apoptotic signalling, determined by Western blotting. Significant induction of cleaved caspase-3 expression was observed in control cells expressing GADD34ΔN but not in mock control cells or control cells infected with AdEmpty-EGFP (**Figure 4.3a**). Cleaved caspase-3 expression was also induced in all cells treated with thapsigargin, with an additive effect observed in GADD34ΔN expressing cells (**Figure 4.3a**). The effect of chronic eIF2α dephosphorylation on cell survival was also determined by measuring the percentage of cells in the sub-G0/G1 (apoptotic) population using flow cytometry. Thapsigargin induced a significant increase in sub-G0/G1 population in mock, AdEmpty and AdGADD34ΔN infected cells in comparison to their respective controls (**Figure 4.3b**). Significant increase in sub-G0/G1 population was however, also observed in unstressed control MIN6 cells expressing GADD34ΔN in comparison to control cells expressing AdEmpty-EGFP (**Figure 4.3b**). The inhibition of eIF2α phosphorylation also had an additive effect on thapsigargin induced apoptosis in MIN6 cells as there was an increase in the sub-GO/G1 population in GADD34ΔN expressing cells treated with thapsigargin in comparison to control GADD34ΔN expressing MIN6 cells (**Figure 4.3b**). The increase in cleaved caspase-3 expression and the increase in sub-G0/G1 population in untreated GADD34ΔN expressing MIN6 cells suggest that basal eIF2α phosphorylation is important for cell survival.

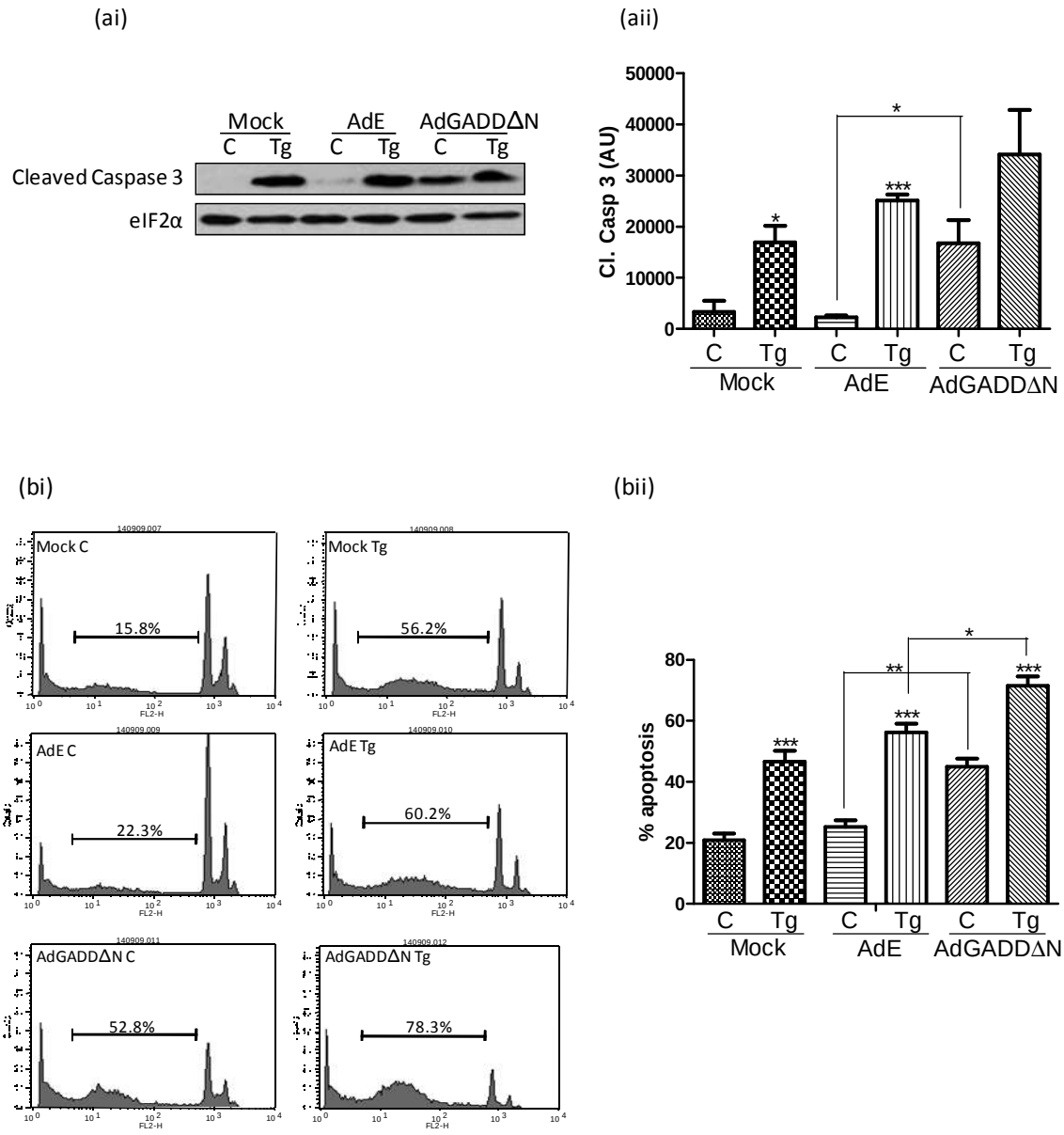


Figure 4.3. Inhibition of eIF2α phosphorylation induces cell death in MIN6 cells. MIN6 cells were mock infected, infected with AdEmpty or AdGADD34ΔN for 24 h. Following infection, cells were treated with 1μM thapsigargin for a further 24 h. (ai) Cells were lysed and proteins resolved on SDS-PAGE and Western blotted using antisera against cleaved caspase 3 and total eIF2α as a loading control. (aii) Densitometric analysis of cleaved caspase 3 expression is shown in arbitrary units. (bi) After treatment time elapsed, cells were collected using trypsin and fixed overnight in methanol. Cells were then stained with propidium iodide for 30 min at RT and cell cycle analysis carried out using flow cytometry. (bii) Apoptotic cells were quantified and presented as a graph. Results shown are either representative or expressed as mean ± SEM of at least three independent experiments. Statistical analysis was carried out using an unpaired two-tailed Students' t-test. *, P<0.05; **, P<0.01; ***, P<0.001 *vs*. control.

4.2.4 Inhibition of eIF2α phosphorylation has no effect on thapsigargin induced signalling via the IRE1 pathway.

Harding et al., reported that the IRE1α arm of the UPR is activated in PERK-deficient cells to compensate for the loss of PERK function in mouse embryonic stem cells (Harding, Zhang et al. 2000). To investigate whether the suppression of PERK-dependent eIF2α phosphorylation in thapsigargin treated β-cells caused an increase in IRE1α activity, MIN6 and INS1E cells were infected with AdEmpty-EGFP or AdGADD34ΔN prior to treatment with thapsigargin. Changes in the phosphorylation state of IRE1α and its activity through monitoring changes in the expression of spliced XBP1 were determined by Western blotting. The suppression of eIF2α phosphorylation had no effect on the phosphorylation state of IRE1α in MIN6 (**Figure 4.4a**) nor INS-1E (**Figure 4.4b**) cells. XBP1 splicing was also unaffected by the inhibition of eIF2α phosphorylation in MIN6 (**Figure 4.4a**) and INS-1E (**Figure 4.4b**) cells.

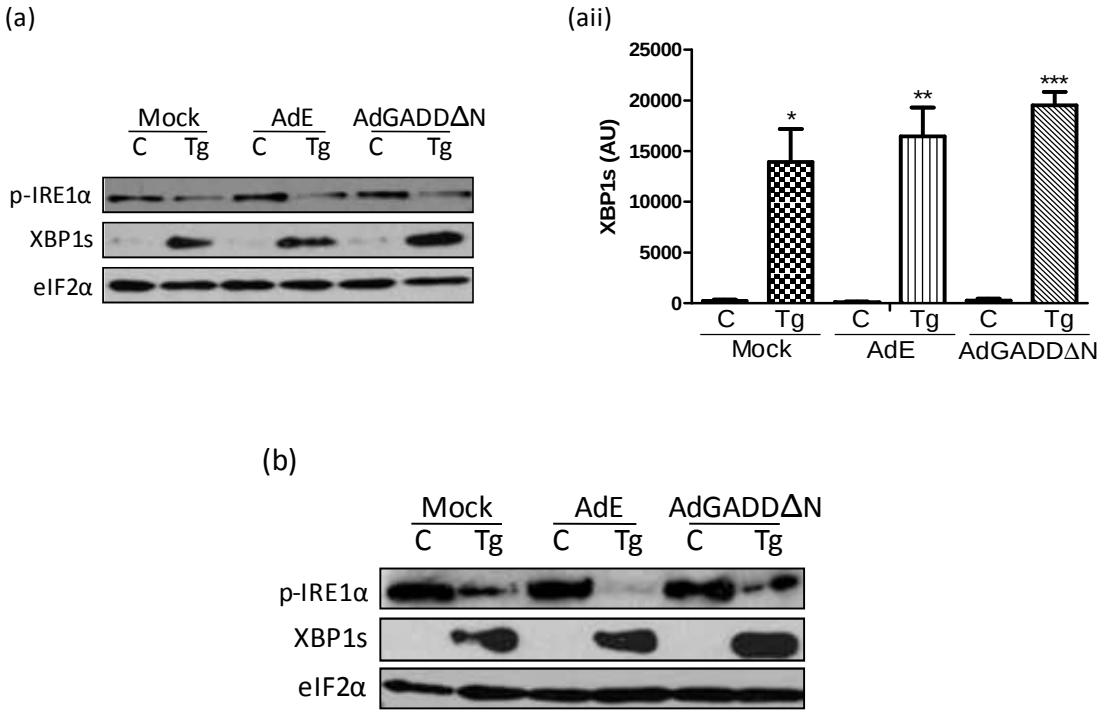


Figure 4.4. Inhibition of eIF2α phosphorylation has no effect on thapsigargin induced IRE1 signalling. (a) MIN6 cells or (b) INS-1E cells were mock infected, infected with AdEmpty or AdGADD34ΔN for 24 h. Following infection, cells were treated with 1μM thapsigargin for a further 24 h. Cells were lysed and proteins resolved on SDS-PAGE and Western blotted using antisera against phosphorylated IRE1α and total eIF2α as a loading control. Nuclear extracts were resolved on SDS-PAGE and Western blotted using antisera against spliced XBP1. (aii) Densitometric analysis of XBP1s is shown for

MIN6 cells in arbitrary units. MIN6 results shown are either representative or expressed as mean ± SEM of at least three independent experiments. Experiment for nuclear extract in INS-1E cells n=1. Statistical analysis was carried out using an unpaired two-tailed Students' t-test. *, P<0.05; **, P<0.01; ***, P<0.001 *vs*. control.

4.2.5 Inhibition of eIF2α phosphorylation has no effect on the downstream expression of ATF4 in palmitate treated MIN6 cells.

Results obtained so far in this study indicate that the forced dephosphorylation of eIF2α under thapsigargin induced ER stress conditions is detrimental to cell survival and did not protect cells from ER stress induced cell death. It was also observed that ER stress mediated expression of ATF4 and CHOP was unaffected by the suppression of eIF2α phosphorylation. To investigate the effects of chronic eIF2α dephosphorylation in MIN6 cells under more physiological ER stress conditions, the saturated fatty acid, palmitate was used to induce ER stress in GADD34ΔN expressing MIN6 cells. Characterisation of palmitate induced ER stress and cell death in MIN6 cells was determined in Chapter 3. Based on these results, MIN6 cells were either mock infected, infected with AdEmpty-EGFP or AdGADD34ΔN for 24 h prior to treatment with 0.5mM palmitate for 2, 12 and 24 h. As expected, palmitate induced eIF2α phosphorylation by 2 h and 12 h of treatment in mock and AdEmpty infected MIN6 cells in comparison to the appropriate controls (**Figure 4.5a, b**). Also expectedly, no significant eIF2α phosphorylation was observed at 24 h of palmitate treatment in comparison to controls (**Figure 4.5c**). The expression of GADD34ΔN inhibited both basal and palmitate induced eIF2α phosphorylation in MIN6 cells at all time points tested (**Figure 4.5**). The expression of ATF4 and CHOP was assessed at 24 h. In correlation with the observations made in Chapter 3, palmitate treatment resulted in increased expression of ATF4 and CHOP in mock infected and AdEmpty infected MIN6 cells (**Figure 4.5c**). The suppression of eIF2α phosphorylation in palmitate treated GADD34ΔN expressing MIN6 cells did not inhibit the expression of ATF4 or CHOP (**Figure 4.5c**).

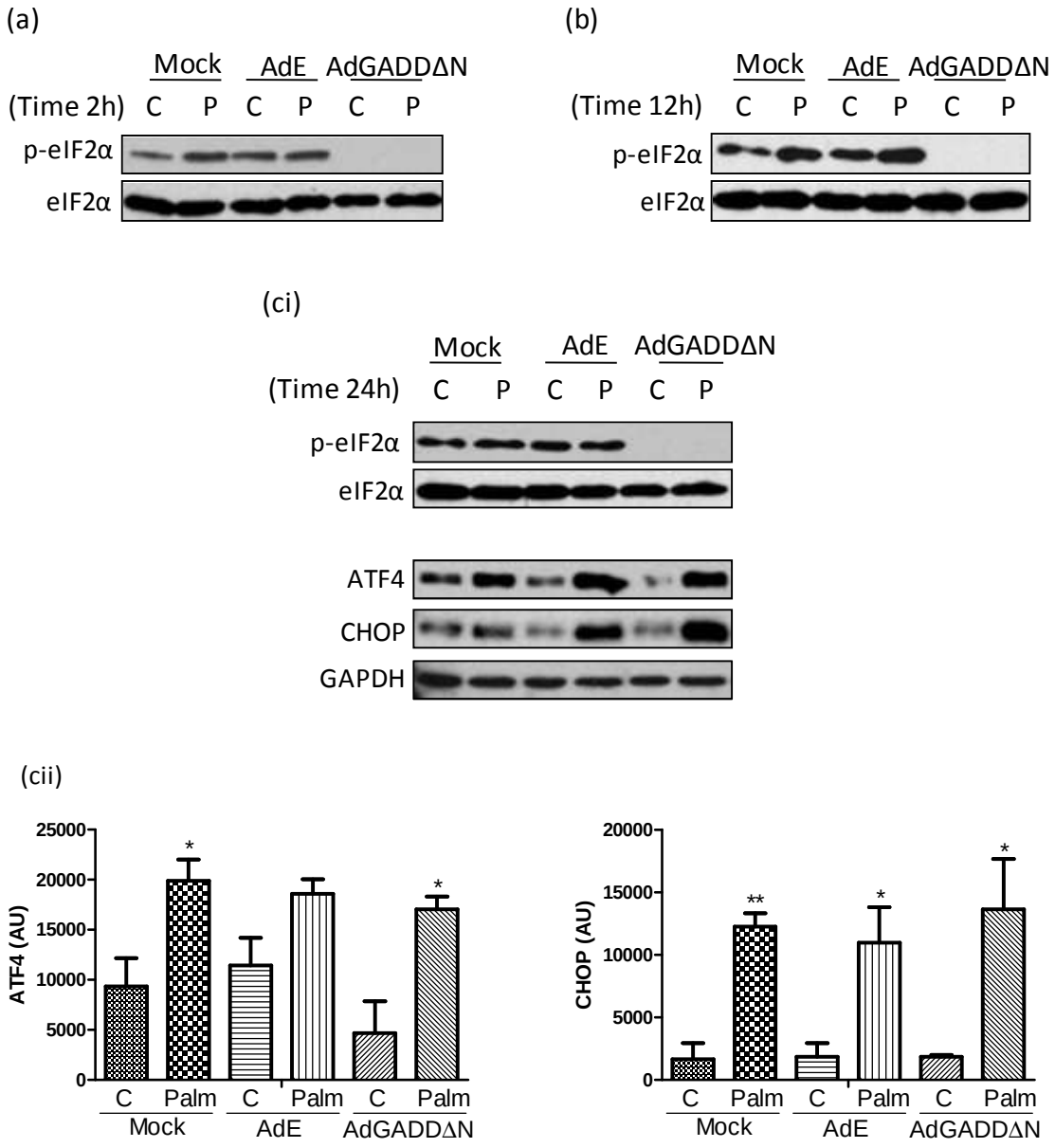


Figure 4.5. Inhibition of palmitate induced eIF2α phosphorylation by AdGADD34ΔN does not inhibit the downstream expression of ATF4 and CHOP. MIN6 cells were mock infected, infected with AdEmpty or AdGADD34ΔN for 24 h. Following infection, cells were treated with 0.5mM palmitate or control cells were treated with 0.67% BSA and a similar dilution of ethanol for a further (a) 2 h, (b) 12 h or (c) 24 h. Cells were lysed and proteins resolved on SDS-PAGE and Western blotted using antisera against p-eIF2α and total eIF2α as a loading control. Nuclear extracts were resolved on SDS-PAGE and Western blotted using antisera against ATF4 and CHOP. GAPDH was detected as a loading control. (cii) Densitometric analysis of ATF4 and CHOP expression at 24 h is shown in arbitrary units and expressed as mean ± SEM of at least three experiments. Blots shown for 24 h experiment are representative of at least three experiments. 2 h and 12 h experiments; n=1. Statistical analysis was carried out using an unpaired two-tailed Students' t-test. *, P<0.1; **, P<0.01; ***, P<0.001 *vs* control.

4.2.6 Inhibition of eIF2α phosphorylation induces cleaved caspase-3 expression in palmitate treated MIN6 cells.

Caspase-3 is an effector of apoptosis which is activated upon proteolytic cleavage (Morishima, Nakanishi et al. 2002). Chronic dephosphorylation of eIF2α resulted in the increased detection of cleaved caspase-3 and an increase in the population of apoptotic cells in thapsigargin treated MIN6 cells (**Figure 4.3**). To investigate whether inhibition of eIF2α phosphorylation in palmitate treated cells also resulted in increased cleaved caspase-3 expression, MIN6 cells infected with AdEmpty (control cells) or AdGADD34ΔN were treated with palmitate for 24 h and the expression of cleaved caspase-3 assessed by Western blotting. Palmitate induced an increase in caspase-3 cleavage in mock and AdEmpty infected MIN6 cells from 12 h in comparison to the corresponding controls (**Figure 4.6b,c**). The chronic suppression of eIF2α phosphorylation in GADD34ΔN expressing MIN6 cells however induced the cleavage of caspase-3 in both control and palmitate treated cells at all time points assessed (**Figure 4.6**). This result confirms that inhibition of both basal and ER stress induced eIF2α phosphorylation results in cleaved caspase-3 expression, an indicator of apoptotic signalling.

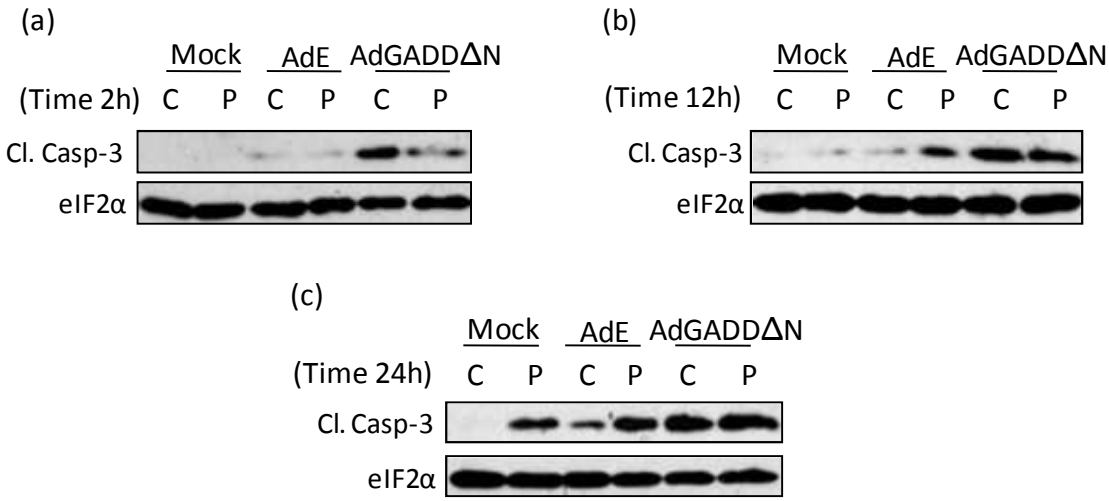


Figure 4.6. Inhibition of eIF2α phosphorylation induces cleaved caspase-3 expression in MIN6 cells. MIN6 cells were mock infected, infected with AdEmpty or AdGADD34ΔN for 24 h. Following infection, cells were treated with 0.5mM palmitate for a further (a) 2 h (b) 12 h or (c) 24 h. Control cells were treated with 0.67% BSA and a similar dilution of ethanol for the same duration. Cells were lysed and proteins resolved on SDS-PAGE and Western blotted using antisera against cleaved caspase-3 and total eIF2α as a loading control. n=1.

4.2.7 Inhibition of eIF2α phosphorylation mildly induces partial inhibition of IRE1 phosphorylation but has no effect on XBP1 splicing in palmitate treated MIN6 cells.

To determine whether the inhibition of palmitate mediated eIF2α phosphorylation results in the activation of the IRE1 signalling pathway, mock, AdEmpty or AdGADD34ΔN infected cells were treated with palmitate for 24 h and the expression of phosphorylated IRE1 and XBP1 splicing was assessed by Western blotting and RT-PCR respectively. At 24 h of palmitate exposure, the phosphorylation of IRE1 in AdGADD34ΔN expressing MIN6 cells was reduced in comparison to AdEmpty infected cells (**Figure 4.7**). In AdGADD34ΔN expressing cells however, palmitate caused a slight increase in IRE1 phosphorylation in comparison to untreated control cells (**Figure 4.7**). The splicing of XBP1 downstream of IRE1 activation was also assessed. The inhibition of eIF2α phosphorylation did not have any significant effect on the splicing of XBP1 in response to a 24 h palmitate exposure (**Figure 4.7**). These results suggest that the inhibition of eIF2α phosphorylation has no significant effect on the activation of and signalling downstream of the IRE1α pathway.

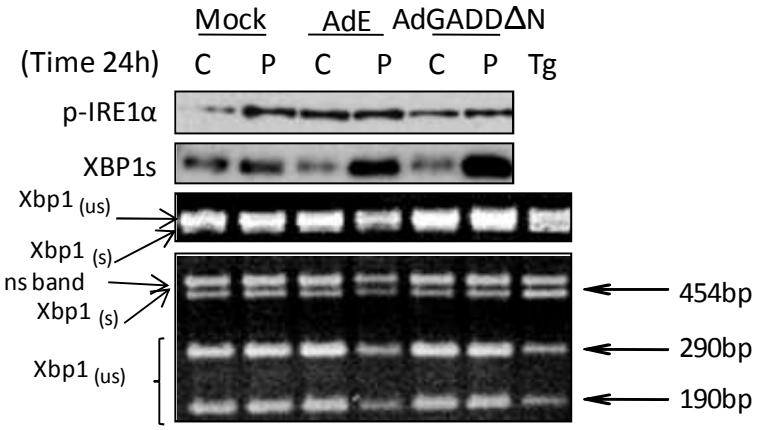


Figure 4.7. AdGADD34ΔN mediated eIF2α dephosphorylation induces a mild IRE1α dephosphorylation in palmitate treated MIN6 cells but has no effect on XBP1 splicing. MIN6 cells were mock infected, infected with AdEmpty or AdGADD34ΔN for 24 h. After infection, cells were treated with 0.5mM palmitate for a further 24 h. Control cells were treated with 0.67% BSA and a similar dilution of ethanol for the same duration. After experiment time elapsed, cells were lysed and proteins resolved on SDS-PAGE and Western blotted using antisera against p-IRE1α. Nuclear extracts were resolved on SDS-PAGE and Western blotted using antisera against spliced XBP1. Total RNA was also extracted from cells using Trizol. After reverse transcription, XBP1 cDNA

was amplified by PCR followed by incubation with Pst1 which cuts the PCR products produced from unspliced XBP1 into two smaller fragments of 290bp and 190bp. Spliced XBP1 (454bp) lacks the Pst1 restriction site and remains intact. Blots shown are representative of at least two independent experiments.

4.3 Discussion

Chronic activation of the UPR has been implicated in the gross loss of β-cell mass by apoptosis in type 2 diabetes (Kharroubi, Ladriere et al. 2004, Karaskov, Scott et al. 2006, Wang, Kouri et al. 2005). In Chapter 3, I showed that chronic induction of ER stress by thapsigargin and palmitate results in the loss of cell viability and induction of apoptosis in MIN6 cells as evidenced by cleaved caspase-3 expression. The signalling mechanism by which ER stress causes the induction of apoptosis in these cells is not fully understood. I also show in Chapter 3 that CHOP expression is upregulated after chronic exposure of MIN6 cells to both thapsigargin and palmitate. Increased CHOP expression has been associated with the induction of apoptosis and CHOP expression has been reported to be transcriptionally up-regulated by ATF4 whose expression is increased in response to eIF2α phosphorylation (Okada, Yoshida et al. 2002) and others. It is therefore possible that ER stress induced apoptosis is mediated by PERK/p-eIF2α dependent increases in CHOP expression. In this study, I aimed to determine the role of PERK dependent eIF2α phosphorylation in chronic ER stress induced cell death. Here, I provide evidence that eIF2α phosphorylation is not required for ER stress induced cell death and that chronic dephosphorylation of eIF2α is detrimental to cellular survival in MIN6 cells. I also provide evidence which suggests that the expression of ATF4 and CHOP under ER stress conditions may be additionally regulated by a novel pathway independent of eIF2α phosphorylation.

4.3.1 Chronic eIF2α dephosphorylation is detrimental to cell survival

The PERK-eIF2α branch of the UPR has been proposed to be crucial for the maintenance of cellular functional integrity by mediating recovery from ER stress through the inhibition of protein translation to match folding capacity within the ER (Jousse, Oyadomari et al. 2003, Harding, Zhang et al. 2000). In particular, the phosphorylation of eIF2α has been suggested to promote β-cell survival in several studies (Harding, Zeng et al. 2001, Scheuner, Song et al. 2001, Scheuner, Mierde et al. 2005). The transient nature of eIF2α phosphorylation under ER stress conditions is particularly suggested to be important for β-cell survival as the deletion of p58, an ER chaperone protein which is induced and negatively regulates PERK under ER stress

conditions (Yan, Frank et al. 2002), resulted in the development of diabetes and pancreatic β-cell apoptosis in p58-null mice (Ladiges, Knoblaugh et al. 2005). In addition, chronic phosphorylation of eIF2α induced by salubrinal under ER stress conditions in INS-1E cells was found to be detrimental to cell survival (Cnop, Ladriere et al. 2007), supporting a role for transient eIF2α phosphorylation in cell survival.

In my study, the chronic dephosphorylation of eIF2α in control GADD34ΔN expressing MIN6 cells was found to induce significant cell death in comparison to AdEmpty infected control cells **(Figure 5.3)**. Similar inhibition of eIF2α phosphorylation in immortal mouse hippocampal (HT22) cells was also reported to compromise cell survival in unstressed cells (Lu, Jousse et al. 2004). This observation, in agreement with my study, suggests that the basal phosphorylation state of eIF2α serves a role in the survival and function of β-cells. It has additionally been reported that the inhibition of eIF2α phosphorylation in mice resulted in unregulated proinsulin translation, increased oxidative damage and apoptosis (Back, Scheuner et al. 2009), suggesting that basal eIF2α phosphorylation may also be required to prevent excessive protein synthesis in the β-cell thereby limiting the possibility of the accumulation of unfolded proteins or oxidative stress. Moreover, eIF2α phosphorylation has been reported to maintain the expression of Pdx1 and MafA, transcription factors which are necessary for β-cell differentiation, proliferation and survival (Ahlgren, Jonsson et al. 1998, Ackermann, Gannon 2007, Kaneto, Miyatsuka et al. 2008). In consideration of this, chronic suppression of eIF2α phosphorylation in control cells would therefore result in unlimited protein synthesis and an increase in the possibility of the accumulation of unfolded proteins, chronic ER stress and induction of apoptosis in the β-cells. In agreement with several other reports (Scheuner, Song et al. 2001, Zhang, McGrath et al. 2002, Harding, Zhang et al. 2000), the chronic inhibition of eIF2α phosphorylation in our MIN6 cells was also found to render the cells more susceptible to stress. Thus, treatment of GADD34ΔN expressing MIN6 cells with thapsigargin or palmitate resulted in the potentiation of apoptosis in these cells (**Figures 4.3, 4.6**). The accumulation of unfolded proteins which may occur under conditions of suppressed eIF2α phosphorylation, coupled with additive stress by the ER stress inducers may therefore result in the observed potentiation of apoptosis.

The reduction in cell survival under conditions of impaired PERK signalling has been proposed to occur as a result of a parallel and compensatory increase in the activation of the IRE1 pathway (Harding, Zhang et al. 2000). IRE1 has been shown to couple ER stress signals to apoptosis by inducing the activity of JNK, a pro-apoptotic kinase (Urano, Wang et al. 2000). In this study however, the inhibition of eIF2α phosphorylation in ER stressed MIN6 cells had no compensatory effect on the activation of IRE1 as assessed by XBP1 splicing (**Figures 4.4, 4.7**). This suggests that IRE1 signalling has no significant effect on the decrease in cell survival observed in MIN6 cells expressing GADD34ΔN.

Taken together, the observations made in this study and in published reports by others, suggest that the phosphorylation and dephosphorylation of eIF2α in UPR signalling is subject to very delicate fine tuning and that any manipulation of the kinase and/or phosphatase activities within this pathway results in the activation of an apoptotic signalling program in the β-cell.

4.3.2 ATF4 and CHOP expression is induced under ER stress conditions independent of eIF2α phosphorylation

An interesting observation in this study was the persistent upregulation of ATF4 and CHOP protein expression under ER stress conditions even in the absence of eIF2α phosphorylation (**Figures 4.2, 4.5**). This is in contrast to reports in which the protein expression of ATF4 and CHOP was inhibited in MEF cells homozygous for a mutation in the Ser51 residue (Ser51Ala) (Scheuner, Song et al. 2001; Lu, Jousse et al. 2004). In those studies, pharmacological induction of ER stress in the mutant MEF cells did not stimulate ATF4 and CHOP protein expression despite an increase in the corresponding mRNA. The reason for this difference in results is unclear but may be due to the fact that my study was carried out in clonal β-cell lines while the above mentioned studies were in MEF cells. While the cause is unknown, differences in ER stress signalling between different cell types have previously been reported (Cnop, Ladriere et al. 2007).

Under ER stress conditions, Harding et al reported that the translational upregulation of ATF4 is dependent on the phosphorylation state of eIF2α (Harding, Novoa et al.

2000b). The authors of that study stated however, that the possibility of other unidentified mechanisms regulating ATF4 translation other than eIF2α phosphorylation had not been excluded as deletion of the first uORF in the ATF4 5' UTR resulted in an increase in translation initiation at the start codon (Harding, Novoa et al. 2000b). Indeed, the expression of ATF4 and CHOP in the absence of eIF2α phosphorylation in my study suggests that a separate novel signalling pathway may be involved in the upregulation of ATF4 protein translation under ER stress conditions. While the upregulation of ATF4 under ER stress conditions is dependent on translation, CHOP expression under the same conditions is regulated at the level of transcription (Harding, Novoa et al. 2000b). In addition to transcription and translation however, protein expression is also regulated by mRNA and protein stability. ATF4 and CHOP mRNA and protein have been reported to be inherently unstable and are thus, rapidly degraded (Rutkowski, Arnold et al. 2006). One may therefore suggest that the expression of ATF4 and CHOP, irrespective of eIF2α phosphorylation, is induced by an actively perpetuating signalling pathway which is independent of eIF2α phosphorylation.

As a result of the dependency of ATF4 expression on PERK activation, it is reasonable to suggest that the proposed alternative pathway in the regulation of ATF4 expression may occur downstream of PERK. It is therefore proposed, that this alternative pathway may be dependent on the activity of the only other known PERK substrate, Nuclear Factor (erythroid-derived 2)-like 2 (Nrf2) (Cullinan, Zhang et al. 2003).PERK has been shown to induce the phosphorylation and subsequent nuclear translocation of Nrf2 under ER stress conditions independent of eIF2α phosphorylation (Cullinan, Zhang et al. 2003, Itoh, Wakabayashi et al. 1999). It may therefore be suggested that despite the inhibition of eIF2α phosphorylation in GADD34ΔN expressing MIN6 and INS-1E cells under ER stress conditions in this study, Nrf2 activation and nuclear translocation may have been induced downstream of PERK activation. In addition to its role in the upregulation of antioxidant enzymes through its interaction with antioxidant response elements (AREs) within the nucleus (Nguyen, Nioi et al. 2009, Kaspar, Niture et al. 2009), Nrf2 may also function to induce the upregulation of ATF4 transcription under ER stress conditions. Indeed, a recent study in retinal pigment endothelial cells

provides evidence for the control of ATF4 gene expression by Nrf2 (Miyamoto, Izumi et al. 2011). In that study, the downregulation of Nrf2 resulted in a concomitant downregulation of ATF4 mRNA in the presence of the ER stress inducer, thapsigargin (Miyamoto, Izumi et al. 2011). The observations in the aforementioned study suggest that the expression of ATF4 under ER stress conditions is at least, partially controlled by the activation of Nrf2 downstream of PERK. Although the activation of PERK was not directly assessed in this study, the phosphorylation state of eIF2α was used as a marker of the activation of the transducer protein. The strategy of targeting the phosphorylation state of eIF2α in this study ensured that the activity of PERK in response to ER stress inducers was unaffected. As such, the phosphorylation and nuclear translocation of Nrf2 downstream of PERK in response to thapsigargin or palmitate treatment would have been unaffected despite the suppression of eIF2α phosphorylation. In view of this, it is hypothesised that the exposure of GADD34ΔN expressing MIN6 and INS-1E cells to thapsigargin or palmitate induced the activation of PERK and the subsequent activation of Nrf2, resulting in the upregulation of ATF4 independent of eIF2α phosphorylation. Nrf2 mediated ATF4 expression would also account for the expression of CHOP which occurs downstream of ATF4 (Fawcett, Martindale et al. 1999).

As previously stated, the expression of ATF4 and CHOP under conditions of suppressed eIF2α phosphorylation in this study is in contrast with several reports in which the inhibition of eIF2α phosphorylation resulted in the downstream repression of ATF4 and CHOP expression (Scheuner, Song et al. 2001; Lu, Jousse et al. 2004). Experiments to investigate the effect of inhibiting Nrf2 activity in cells expressing GADD34ΔN under ER stress conditions may be useful in the determination of the role of Nrf2 in the control of ATF4 expression under ER stress conditions. Alternatively, the possibility that ATF4 expression is co-regulated by a yet to be identified PERK substrate under ER stress conditions cannot be ruled out.

4.3.3 Conclusion

In conclusion, the findings in this chapter demonstrate that chronic eIF2α dephosphorylation is poorly tolerated by β-cells and augments ER stress induced apoptosis. In consideration with other published findings, when designing novel therapies designed to protect β-cells in type 2 diabetes, it is apparent that interference with the PERK-eIF2α arm of UPR signalling may be problematic and potentially harmful. In addition, my findings demonstrate that ATF4 and CHOP upregulation in response to ER stress can occur independently of eIF2α phosphorylation. The mechanism by which this occurs is unclear. Further studies into the alternative mechanisms through which ATF4 expression and thus, CHOP may be regulated may prove useful in the development of new therapies to protect β-cells in type 2 diabetes.

CHAPTER 5

Chapter 5: ER stress signalling in animal models of obesity and diabetes

5.1 Introduction

5.1.1 Obesity and type 2 diabetes

Obesity, insulin resistance and type 2 diabetes belong to a cluster of pathologies known as the metabolic syndrome (Reaven 1991; Kraja, Province et al. 2008). Type 2 diabetes is a metabolic disease characterised by hyperglycaemia in the context of insulin resistance and β-cell dysfunction (Mathis, Vence et al. 2001, Rhodes 2005, Kahn 2003). Insulin resistance is characterised by a decrease in insulin action on glucose uptake and metabolism in peripheral tissues (Porte, Kahn 2001, Polonsky, Sturis et al. 1996) and is generally thought to occur prior to the onset of type 2 diabetes (Martin, Warram et al. 1992). Several lines of evidence however suggest that β-cell dysfunction and in some cases, loss of β-cell mass are pre-induced or induced in parallel with the development of insulin resistance (Kahn 2001; Bagust, Beale 2003; Mitrakou, Kelley et al. 1992). Obesity, the major causal factor of insulin resistance, is also tightly linked to the development of diabetes (Zeyda, Stulnig 2009; Ferrannini, Barrett et al. 1983; Lillioja, Bogardus et al. 1985). Indeed, more than 80% of all type 2 diabetics are reportedly obese with obese individuals often exhibiting elevated plasma free fatty acid (FFA) levels (Goh, Mason et al. 2007; Boden 1997). Obesity alone however, does not account for the development of type 2 diabetes as most obese individuals are capable of maintaining a euglycaemic state despite the presence of insulin resistance (Prentki & Nolan 2006; Rhodes 2005). The maintenance of normoglycaemia in the presence of insulin resistance in obese individuals is facilitated by a compensatory increase in the insulin secretory function of the β-cell through an increase in mass by hypertrophy and an increase in replication and neogenesis (Steil, Trivedi et al. 2001, Liu, Jetton et al. 2002, Chen, Hosokawa et al. 1994, Unger, Orci 2001). Evidence for the increase in β-cell mass in obese insulin-resistant non-diabetic human subjects was published in a study by Butler et al., (Butler, Janson et al. 2003a). In the subset of obese individuals that develop type 2 diabetes, β-cell compensation proves insufficient

to overcome time dependent increases in obesity and insulin resistance resulting in β-cell dysfunction and apoptosis (Butler, Janson et al. 2003a, Unger, Orci 2001, Weir, Laybutt et al. 2001). Autopsies in a small number of type 2 diabetics revealed a reduction in β-cell mass in comparison to both lean and obese non-diabetic subjects (Butler, Janson et al. 2003a, Deng, Vatamaniuk et al. 2004). Amongst other possible factors, elevated levels of circulating glucose and free fatty acids (FFAs) may contribute to the development of β-cell dysfunction and apoptosis (Paris, Bernard-Kargar et al. 2003, Poitout, Robertson 2002). In particular, the induction of chronic ER stress in response to high levels of circulating FFAs as a result of obesity has been implicated in the development of β-cell dysfunction and apoptosis in type 2 diabetes (Prentki & Nolan 2006; Cnop, Welsh et al. 2005; Kahn, Hull et al. 2006). Chronic exposure of clonal β-cell lines to FFAs has been shown to induce ER stress signalling and apoptosis (Kharroubi, Ladriere et al. 2004; Cunha, Hekerman et al. 2008; Laybutt, Preston et al. 2007; Karaskov, Scott et al. 2006). Evidence of ER stress has also been reported in islets isolated from human subjects and animal models of obesity and diabetes (Laybutt, Preston et al. 2007; Huang, Lin et al. 2007; Marchetti, Bugliani et al. 2007).

5.1.2 Evidence of ER stress in the β-cells in obesity and diabetes

In order to determine the contribution of ER stress to the development of β-cell dysfunction and apoptosis in type 2 diabetes and obesity, several studies have assessed the expression of ER stress markers in the islets of human subjects and animal models of obesity and diabetes. The induction of ER stress was reported in the islets of the diabetic *db/db* mice in a study by Laybutt et al., (Laybutt, Preston et al. 2007). The *db/db* mouse possesses an autosomal recessive mutation in the gene encoding the leptin receptor. It is therefore resistant to the appetite suppressing effects of leptin and develops marked obesity, hyperinsulineamia, hyperlipidemia and hyperglycaemia by 8-12 weeks of age (Zhou, Berggren et al. 1996, Tuman, Doisy 1977). Type 2 diabetes develops in the *db/db* mouse as a result of insufficient β-cell compensation for time-dependent increases in obesity and insulin resistance (Zhou, Berggren et al. 1996, Shafrir, Ziv et al. 1999). The mRNA expression of ER stress markers such as ATF4, HSPA5, EDEM1, PDIA4 and DDIT3/CHOP were all significantly upregulated in the islets of the diabetic (*db/db*) mice in comparison to control (*db/+*)

islets (Laybutt et al. 2007). The upregulation of ER stress markers has also been reported in islets and pancreas sections obtained from diabetic human subjects in comparison to non-diabetic controls (Laybutt, Preston et al. 2007, Marchetti, Bugliani et al. 2007). Significant increase in the intensity of islet immunostaining was reported for the ER stress markers HSPA5, DDIT3/CHOP, DNAJC3 and BAX in islets from diabetic human subjects in comparison to islets from non-diabetic subjects (Laybutt, Preston et al. 2007). The induction of HSPA5 and XBP1 was also reported when islets isolated from type 2 diabetics were exposed to high glucose concentrations in comparison to islets isolated from non-diabetic subjects (Marchetti, Bugliani et al. 2007). While Laybutt et al., did not comment on whether the diabetic subjects used in their study were obese, Marchetti et al., reported a higher BMI in the type 2 diabetic subjects in comparison to the non-diabetic donors (Marchetti, Bugliani et al. 2007). An increase in immunostaining for ATF3 was also reported in the islets of human diabetic subjects in comparison to non-diabetic subjects and in the islets of non-obese diabetic mice in comparison to their age-matched wild type littermates (Hartman, Lu et al. 2004). Increased immunostaining for CHOP has also been reported in the islets of both obese non-diabetic and obese type 2 diabetic human subjects in comparison to lean non-diabetic human subjects (Huang, Lin et al. 2007). The nuclear localisation of CHOP was however observed in the islets of obese diabetic subjects in comparison to perinuclear localisation in the islets of the obese non-diabetic and lean non-diabetic human subjects (Huang, Lin et al. 2007). This suggests that while obesity induces the expression of CHOP in the ER stress signalling pathway, other factors present in type 2 diabetes may mediate the translocation and thus, the activation of CHOP function in the islets.

While it is apparent that ER stress is induced in the islets of type 2 diabetics, whether and to what extent ER stress plays a role in the development of β-cell dysfunction is largely unknown (Marchetti, Bugliani et al. 2007). It is also unclear whether ER stress is induced in response to obesity alone, as opposed to a response to both obesity and hyperglycaemia. To investigate this, this study was designed to assess the induction of ER stress in the islets of animal models of obesity and to further assess ER stress when

β-cell dysfunction was induced. Genetic rat models of obesity and/or diabetes as well as diet induced obese mice were employed in this study.

5.1.3 Genetic rodent models of obesity and diabetes

5.1.3.1 Zucker fatty (fa/fa) rats

Zucker Fatty/Obese (ZO) rats possess an autosomal recessive mutation on the gene encoding the leptin receptor (fa/fa) resulting in insulin resistance with reduced glucose tolerance and obesity (Clark, Palmer et al. 1983). Leptin is a hormone produced predominantly in white adipose tissue which directly suppresses insulin secretion through a feedback mechanism while inducing satiation (Kulkarni, Wang et al. 1997). The mutation in the Zucker Obese rat results in hyperphagia, hyperleptinaemia, insulin resistance and obesity due to a reduction in leptin signalling (Clark, Palmer et al. 1983). Obesity in the ZO rats is apparent at approximately 3 weeks of age and corresponds with the onset of robust hyperphagia and hyperinsulineamia (Truett, Walker et al. 2000, Durham, Truett 2006). As a result of obesity, total serum fatty acids have been reported to reach ten times the level in lean rats in comparison to obese rats. Blood glucose however, remains normal in both obese and lean rats (Zucker, Zucker 1961). In response to obesity and insulin resistance, the β-cell initially increases its mass by hypertrophy to compensate for the increase in demand (Jones, Nugent et al. 2010). Changes in Zucker fatty rat islets have been shown to be consistent with increased β-cell function including islet hypertrophy (Topp, Atkinson et al. 2007, Griffen, Wang et al. 2001).

5.1.3.2 Zucker diabetic fatty (fa/fa) rats

ZDF obese rats were originally derived from selective in-breeding of a strain of hyperglycaemic Zucker rats (Peterson, Shaw et al. 1990). Independent of the leptin mutation, these rats also inherit an autosomal recessive mutation mapped to the insulin promoter which affects their ability to compensate for insulin resistance unlike the Zucker Obese rats (Griffen, Wang et al. 2001). Male ZDF obese rats thus spontaneously develop hyperglycaemia on standard rodent chow diet at approximately 7-10 weeks of age following the loss of β-cell mass through apoptosis

(Peterson, Shaw et al. 1990, Unger 1997, Pick, Clark et al. 1998, Etgen, Oldham 2000). In the male ZDF obese rats, a significant increase in plasma insulin and lipid levels precedes the development of hyperglycaemia (Nugent, Smith et al. 2008). In contrast, female ZDF obese rats exhibit the same phenotype as the ZO rat therefore becoming insulin resistant but maintaining normoglycaemia through β-cell mass hypertrophy (Corsetti, Sparks et al. 2000). Female ZDF obese rats however become hyperglycaemic when placed on a high fat (48% kcal) diet (HFD) due to a marked reduction in plasma insulin concentration (Corsetti, Sparks et al. 2000). The development of β-cell dysfunction and hyperglycaemia in the female ZDF rats when placed on a HFD has many features in common with the progression of human type 2 diabetes including a progressive decline in plasma insulin levels which occurs alongside a simultaneous increase in hyperglycaemia (Corsetti, Sparks et al. 2000). A study in which moderate (29.5%) fat content was fed to female ZDF rats also resulted in moderate hyperglycaemia with a rapid decrease in insulin production after 2 weeks and loss of normal islet morphology (Zhou, Madjidi et al. 2005). The ability to selectively induce β-cell dysfunction and hyperglycaemia in the female ZDF obese rats was employed in this study to assess the role of ER stress induction in the pathogenesis of obesity associated type 2 diabetes. It must however be noted here that the female diabetic ZDFO-HFD rat is not a definitive replica of human type 2 diabetes as humans develop spontaneous diabetes which does not occur in this animal model.

5.1.4 Diet Induced model of obesity

5.1.4.1 Diet induced obese (DIO) mice

The diet induced obese (DIO) mouse is a model of obesity and impaired glucose tolerance originally introduced by Surwit et al., in 1988 (Surwit, Kuhn et al. 1988). Obesity is induced in C57B16/J mice by placing 4-8 week old mice on a high fat (40-50% kcal) diet for an extended period of time (Winzell, Ahrén 2004). DIO mice develop insulin resistance as a result of obesity and may also develop insufficient islet compensation resulting in mild hyperglycaemia (Ahrén, Pacini 2002). DIO mice have been reported to be progressively hyperinsulineamic, suggesting progressive worsening of insulin resistance during high fat feeding (Winzell, Ahrén 2004).

5.1.5 Aims

The aim of this study was to characterise ER stress/induction of the UPR in the islets of animal models of obesity. Furthermore, it was to determine if the induction of ER stress in obesity and diabetes correlated with the development of β-cell dysfunction. The induction of ER stress in obesity was assessed in the islets of male Zucker Obese rats, female ZDF Obese rats and DIO mice. ER stress in the progression from obesity into diabetes was assessed in the islets of female ZDF Obese rats placed on a high fat diet to induce β-cell dysfunction. To initially assess the induction of ER stress genes in the islets of the animal models, the mRNA expression of a wide range of genes (**Table 5.1**) was assessed using Taqman Low Density Array (TLDAs) analysis. Other genes including markers of β-cell function and differentiation, markers of lipid handling and metabolism and markers of apoptosis were also assessed to determine β-cell compensation and function in response to obesity and diabetes. Further single gene analysis was carried out thereafter on genes with putative functions downstream of UPR signalling. Raw data and data analysis of genes assessed using TLDAs and single-gene Taqman RT-qPCR are contained in Appendix 2. Please see attached disk.

Table 5.1: Table showing 44 genes potentially regulated by the ER stress signalling pathway.

Markers of ER stress	Gene	Aliases	Function in ER stress signalling	Ref Seq	Rat assay no
Hypoxia up-regulated 1	Hyou1	orp150/Cab140	Functions as a molecular chaperone and participate in protein folding	NM138867.2	Rn00593982_m1
CCAAT/Enhancer binding protein, beta	C/EBPβ	LAP/TF5/NF-IL6	Potentially upregulated under ER stress conditions	NM024125.4	Rn00824635_s1
Wolframin	Wfs1	DFNA14	Participates in the regulation of cellular Ca(2+) homeostasis in the ER	NM031823.1	Rn00582735_m1
DnaJ (Hsp40) homolog, subfamily B, member 9	Dnajb9	ERdj4	Co-chaperone protein upregulated in response to palmitate treatment	NM012699.2	Rn00562259_m1
Nucleobindin	NUCB1		Expressed downstream of IRE1, inhibits cleavage/activation of ATF6	NM053463.1	Rn00584973_m1
Protein disulphide isomerase family A, member 3	Pdia3	Erp57/GRP58	Chaperone protein, induced at low glucose levels	NM017319	Rn00569027_m1
Paraoxonase 2	Pon2		Involved in lipid oxidation and redox reactions in the ER	NM001013082.1	Rn01456019_m1
Protein disulphide isomerase family A, member 4	Pdia4	ERP72/ERP70	Isomerase involved in disulphide bond formation in the ER	NM053849.1	Rn00587766_m1
Calnexin	Canx		Lectin involved in protein quality control before transport away from the ER	NM172008.2	Rn00596877_m1
UDP-glucose ceramide glucosyltransferase-like 1	Ugcgl1	UGGT/UGTR	Involved in protein quality control in the ER	NM133596.1	Rn00592293_m1
Calreticulin	Calr		Lectin involved in protein quality control before transport away from the ER	NM022399.2	Rn00574451_m1
Growth arrest and DNA-damage-inducible, alpha	Gadd45a	DDIT1	Mediates the activation of the JNK pathway in UPR signaling	NM024127.2	Rn00577049_m1
Activating transcription factor 5	Atf5	Atfx	Trancriptional activator, may be upregulated in UPR signalling	NM172336.3	Rn00597319_m1
Mitogen-activated protein kinase 8	Mapk8	JNK	Involved in the apoptotic β-cell death via the IRE1 signalling pathway	XM341399.3	Rn01453358_m1
PRKR-like endoplasmic reticulum kinase	Eif2ak3	PERK/PEK	Transducer protein activated under ER stress conditions	NM031599.1	Rn00581002_m1
Heat shock 70 kDa protein 5	Hspa5	BiP/GRP78	Gene encodes for chaperone protein which is a marker of ER stress	NM013083.1	Rn00565250_m1
Growth arrest and DNA-damage-inducible protein	DDIT3	GADD153/CHOP(10)	Transcription factor upregulated downstream of the PERK pathway, induces apoptosis	NM024134	Rn00492098_g1
Myeloid differentiation primary response gene 116	Myd116	GADD34	Induced downstream of ATF4, shown to dephosphorylate eIF2α	NM133546.2	Rn00591894_m1
DnaJ (Hsp40) homolog, subfamily C, member 3	Dnajc3	HSP40, DnaJ, p58	Increases with increase in palmitate mediated apoptosis	NM022232.1	Rn00573712_m1
Activating transcription factor 4	Atf4	CREBP2	Upregulated downstream of PERK activation	NM024403.1	Rn00824644_g1
Homocysteine-inducible, ER stress-inducible	Herpud1	Herp, Mif1	Expression is induced by the UPR, component of the ERAD machinery	NM053523.1	Rn00585371_m1
Tribbles 3	Trib3	SINK/SKIP3	Binds to and inhibits ATF4 transcriptional activation activity	NM144755.2	Rn00595314_m1
X-box binding protein 1	Xbp1	TREB5	Transcription factor activated under UPR signalling	NM001004210.1	Rn01752572_g1
Quiescin Q6 sulfhydryl oxidase 1	Qscn6	qsox1	Isomerase involved in disulphide bond formation in the ER	NM053431.3	Rn00584808_m1
FK506 binding protein 11	FkBP11	FkBP19	Involved in folding of proline-containing polypeptides in the ER	NM001013105.1	Rn01532810_m1
Activating transcription factor 3	ATF3	bZip protein	Upregulated downstream of ATF4, may function in apoptosis signalling	NM012912.1	Rn00563784_m1

Markers of β-cell function & Differentiation	Gene	Aliases	(Potential) Function in ER stress signalling	Ref Seq	Rat assay no
Insulin 2	Ins2		Gene expression is inhibited under ER stress conditions	NM008387	Rn01774648_g1
Cyclin D1	Ccnd1	PRAD1	Synthesis is inhibited by PERK resulting in UPR-associated cell cycle arrest	NM007631	Rn00432359_m1
Glucose transporter type 2	Slc2a2	GLUT2	Glucose transporter, mediates bidirectional transport of glucose	X78722	Rn00563565_m1
Pancreatic and duodenal homeobox 1	Pdx1	MODY4	Protein functions as a transcriptional activator of several genes	NM008814	Rn00755591_m1
Insulin Receptor Substrate 2	Irs2		Protein is phosphorylated by Insulin Receptor, mediates response to insulin	NM001168633.1	Rn01482270_s1
Glucokinase/ Hexokinase 4	Gck	MODY2/HK4	Involved in glucose oxidation in the mitochondria	NM010292	Rn00561265_m1

Markers of lipid handling and metabolism	Gene	Aliases	(Potential) Function in ER stress signalling	Ref Seq	Rat assay no
Sterol Regulatory Element Binding Protein–1	SREBP1	SREBP1/ ADD1	Transcription factor which regulates lipogenic gene expression	XM213329.4	Rn01495763_g1
HMG-CoA reductase	Hmgcr		Involved in Fatty Acid Metabolism , downstream of SREBP	M62766	Rn00565598_m1
Low density lipoprotein receptor	LDLR	FHC	Binds LDL and transports into cells by endocytosis	NM010700	Rn00598438_m1
Fatty Acid Synthase	Fasn	Fas	Catalyzes long-chain fatty acid synthesis	NM007988	Rn00569117_m1
Acetyl CoA Carboxylase	Acaca	ACCA	Involved in Fatty Acid Metabolism, downstream of SREBP	AAG01858	Rn00573474_m1
Free fatty acid receptor 1	Ffar1	GPR40	Gq coupled receptor resident on the β-cell ER membrane	NM153304.1	Rn00824686_s1

Markers of Apoptosis	Gene	Aliases	(Potential) Function in ER stress signalling	Ref Seq	Rat assay no
Bcl-2 homologous antagonist/killer	Bak1	CDN1	involved in IRE1 mediated apoptosis		
B-cell lymphoma protein 2	Bcl2		Anti-apoptotic gene, may be inhibited in UPR signalling	U51278	Rn99999125_m1
Caspase 12	Casp 12		Involved in caspase pathway, apoptotic signalling		Rn00590440_m1
BCL2-associated X protein	Bax		Involved in IRE1 mediated apoptosis, increases in db/db mice	NM007527	Rn02532082_g1
BCL-X/BCL-2 binding protein	BAD	BBC2	Pro-apoptotic gene, binds BClx to inhibit death repressot activity	NM007522	Rn00575519_m1

Miscellaneous	Gene	Aliases	(Potential) Function in ER stress signalling	Ref Seq	Rat assay no
Uncoupling protein 2	Ucp2	SLC25A8	Induced by FFA treatment, may be involved in impairment of glucose action	U69135	Rn01754856_m1

5.2 Results

5.2.1 Obesity correlates with an increase in the expression of markers of ER stress and activation of the UPR in rodent models of obesity

5.2.1.1 Zucker obese rats

To determine whether obesity induces ER stress and the induction of the UPR in islets, changes in gene expression of key markers of ER stress and the UPR (**Table 5.2**) were analysed in Zucker Obese rats in comparison to their lean littermates. In addition, genes encoding proteins with roles in β-cell function and differentiation, lipid handling and metabolism and cellular apoptosis were also analysed. ZO rats are an established model of obesity and the phenotypic and metabolic characteristics of this model have been extensively studied with reports of hyperinsulineamia, insulin resistance, glucose intolerance and hyperlipidemia (Shafrir 1992). By 11-weeks of age, ZO rats weighed significantly more than their lean littermates fed on the same standard chow diet (**Figure 5.1ai**). The obese rats were also hyperinsulineamic with a significant increase in fasting blood glucose in comparison to their lean littermates (**Figure 5.1aii**). Characteristically significant increases in plasma triglycerides and glycosylated haemoglobin, a measure of average plasma glucose concentration, were also observed in the ZO rats in comparison to lean littermates (**Figure 5.1aii**).

Islet RNA was extracted from the rats and the comparative expression levels of 44 genes were assessed using islet specific Taqman Low Density Arrays (TLDAs). This method of gene card array analysis allowed for a high throughput method of analysing changes in the expression of a large number of genes in our model of obesity. In the obese rats, statistically significant increases were observed in the expression of 20 out of the 26 ER stress markers analysed (**Table 5.2**). These included the chaperone proteins, quality control proteins and foldases such as HYOU1, PDIA4, calreticulin, HSPA5, PDIA3, DNAJB9, and calnexin. The expression of the gene encoding the transducer protein PERK (EIF2AK3) and downstream effectors of the PERK pathway such as ATF4, and MYD116/GADD34 were also significantly upregulated in the ZO islets in comparison to lean control islets.

In parallel, changes in the expression of markers of β-cell function and cell cycle progression were also assessed in the islets of the ZO rats in comparison to lean control rat islets. These included insulin-2 (INS2), glucose transporter type 2 (SLC2A2/GLUT2), insulin receptor substrate 2 (IRS2) and glucokinase (GCK) which were all significantly upregulated in the obese rat islets in comparison to lean controls (**Table 5.2**). PDX1, a transcription factor specifically expressed in the β-cell and cyclin D1 (CCND1), involved in cell cycle progression were both significantly upregulated in obese rat islets in comparison to lean controls (**Table 5.2**). This data indicates that there is an attempt to compensate for insulin resistance through an increase in function in the islets of the obese rat model in comparison to their lean littermates.

In addition to the induction of ER stress, the transcriptional response to hyperlipidemia as a result of obesity was also assessed. Obesity resulted in a statistically significant increase in the expression of genes involved in the regulation and metabolism of lipids such as HMG-CoA reductase (HMGCR), sterol regulatory element binding protein-1 (SREBF1), low density lipoprotein receptor (LDLR) and fatty acid synthase (FASN) (**Table 5.2**). FFAR1, the gene encoding GPR40, a free fatty acid receptor predominantly expressed in β-cells was also significantly upregulated. This gene is regulated by PDX1, a transcription factor which was also significantly upregulated (**Table 5.2**).

As obesity has been implicated in the development of insulin resistance, type 2 diabetes and the eventual loss of β-cells through ER stress induced apoptosis (Cnop, Welsh et al. 2005, Kahn 2003), the expression of apoptotic markers was assessed in the islets of the ZO rats in comparison to their lean littermates. Statistically significant increases in the expression of both pro-apoptotic (BAK1, caspase-12, BAX, BAD) as well as anti-apoptotic (BCL2) markers were observed in the islets of the obese rats in comparison to lean controls (**Table 5.2**).

The expression of UCP2, a protein implicated in the decreased secretory response of the β-cell, was also assessed. UCP2 upregulation is reported in MIN6 cells chronically exposed to the free fatty acids oleate and palmitate (Lameloise, Muzzin et al. 2001). In

agreement with this report, a significant increase in the expression of UCP2 was observed in the islets of the ZO rats in comparison to lean control islets (**Table 5.2**).

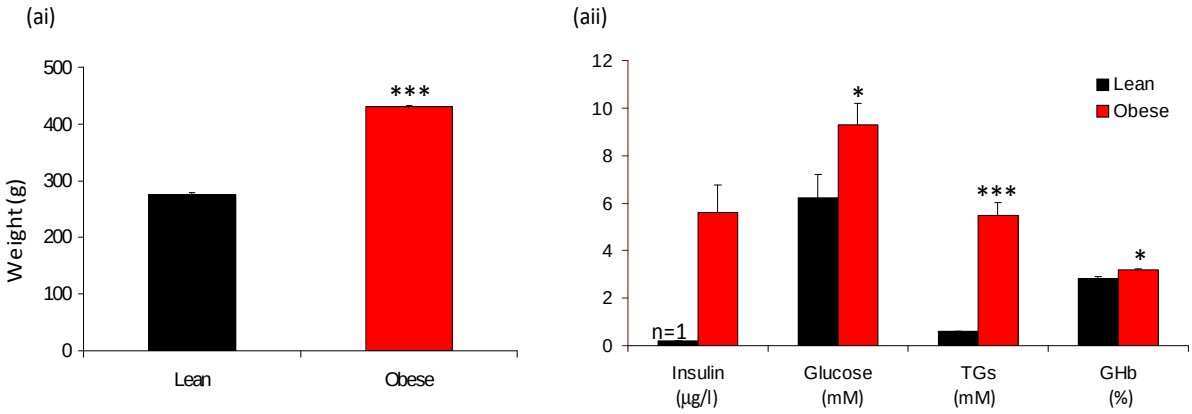


Figure 5.1. Body weights and plasma biochemistry in 11-week old obese *vs.* lean male Zucker rats (ai) Body weight in *grams* and (aii) plasma insulin, blood glucose, plasma triglyceride and glycosylated haemoglobin levels were measured in obese and lean Zucker rats after an overnight fast. Data are shown as mean ± SEM (ZO, n=5; ZL, n=4). Statistical significance was determined using an unpaired two-tailed Student's t-test. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$ *vs.* lean control.

Table 5.2

Islet TLDA gene expression results (male Zucker obese rats).

Data obtained for all genes included on islet TLDA and expressed as fold change compared to control Zucker lean rats. Data is normalised to housekeeping gene 18s.

ER stress genes Gene Name	Gene Symbol	ZO islets
Hypoxia up-regulated 1	Hyou1	4.41
Wolframin	Wfs1	3.51
DnaJ (Hsp40) homolog, subfamily B, member 9	Dnajb9	3.25
CCAAT/Enhancer binding protein, beta	Cebpb	3.25
Nucleobindin	Nucb1	3.17
Protein disulphide isomerase family A, member 3	Pdia3	3.08
Paraoxonase 2	Pon2	3.05
Protein disulphide isomerase family A, member 4	Pdia4	2.95
Calnexin	Canx	2.95
UDP-glucose ceramide glucosyltransferase-like 1	Ugcgl1	2.87
Calreticulin	Calr	2.61
Growth arrest and DNA-damage-inducible, alpha	Gadd45a	2.61
Activating transcription factor 5	Atf5	2.49
Mitogen-activated protein kinase 8	Mapk8	2.41
PRKR-like endoplasmic reticulum kinase	Eif2ak3	2.33
Heat shock 70 kDa protein 5	Hspa5	2.22
Growth arrest and DNA-damage-inducible protein	Ddit3	2.2
Myeloid differentiation primary response gene 116	Myd116	2.03
DnaJ (Hsp40) homolog, subfamily C, member 3	Dnajc3	1.86
Activating transcription factor 4	Atf4	1.65
Homocysteine-inducible, ER stress-inducible	Herpud1	1.47
Tribbles 3	Trib3	1.33
X-box binding protein 1	Xbp1	1.3
Quiescin Q6 sulfhydryl oxidase 1	Qscn6	-1.02
FK506 binding protein 11	Fkbp11	-1.21
Activating transcription factor 3	Atf3	-1.41

β-cell function & differentiation genes Gene Name	Gene Symbol	ZO islets
Insulin 2	Ins2	5.71
Cyclin D1	Ccnd1	4.41
Glucose transporter type 2	Slc2a2/Glut2	4.26
Pancreatic and duodenal homeobox 1	Pdx1	3.26
Insulin Receptor Substrate 2	Irs2	3.25
Glucokinase/ Hexokinase 4	Gck	3.09

Lipid handling and metabolism genes Gene Name	Gene Symbol	ZO islets
Sterol Regulatory Element Binding Protein–1	Srebf1	2.22
HMG-CoA reductase	Hmgcr	3.78
Low density lipoprotein receptor	Ldlr	3.56
Fatty Acid Synthase	Fasn	2.28
Acetyl CoA Carboxylase	Acaca	2.12
Free fatty acid receptor 1	Ffar1	3.14

Apoptosis genes Gene Name	Gene Symbol	ZO islets
Bcl-2 homologous antagonist/killer	Bak1	4.78
B-cell lymphoma protein 2	Bcl2	4.12
Caspase 12	Casp 12	2.80
BCL2-associated X protein	Bax	2.20
BCL-X/BCL-2 binding protein	Bad	2.04

Miscellaneous genes Gene Name	Gene Symbol	ZO islets
Uncoupling protein 2	Ucp2	4.89

5.2.1.1.1 Transcriptional analysis by single gene Taqman RT-qPCR

To confirm the data obtained using the TLDAs, a more stringent assessment of changes in the expression of a selected set of genes was carried out by single gene quantitative RT-PCR. Genes encoding ER stress markers such as calreticulin, HSPA5, HYOU1, PDIA4 and ERO1β were all significantly upregulated in the islets of the ZO rats in comparison to lean control rat islets (**Figure 5.2a**). The significant increase in the expression of these genes indicates the activation of a mechanism involved in the folding and processing of newly synthesised proteins as occurs in the UPR. The expression of other ER stress markers such as GADD45A and NUCB1 was also significantly upregulated in the islets of the ZO rats in comparison to lean control rat islets (**Figure 5.2a**). Under ER stress conditions, ATF4 is translationally upregulated (Harding, Novoa et al. 2000b). There was however, no significant change in the transcriptional expression of ATF4 and the downstream expression of ATF3 in the ZO rat islets in comparison to control rat islets (**Figure 5.2a**). The expression of the EIF4BP gene which encodes the 4EBP1 protein was also assessed. 4EBP1 is a translational repressor which has been reported to be upregulated downstream of ATF4 under ER stress conditions in MIN6 cells (Suguru Yamaguchi, Hisamitsu Ishihara et al. 2008). In contrast, a statistically significant downregulation of EIF4BP was observed in the islets of the ZO rats in comparison to lean control rat islets (**Figure 5.2a**). The transcriptional expression of the INS2 gene was also assessed as a measure of β-cell function. Although not statistically significant, there was a trend towards an increase in the expression of insulin in the islets of the ZO rats in comparison to the lean rat islets (**Figure 5.2b**). The expression of apoptotic markers was also assessed. Statistically significant increases were observed in the expression of caspase-12 and DDIT3/CHOP in the islets of the ZO rats in comparison to lean controls (**Figure 5.2c**). The increase in CHOP expression was accompanied by a similarly statistically significant increase in the expression of its binding partner C/EBPβ (**Figure 5.2c**). Overall, this data confirms the TLDA data and suggests increased β-cell function with a concomitant increase in β-cell mass.

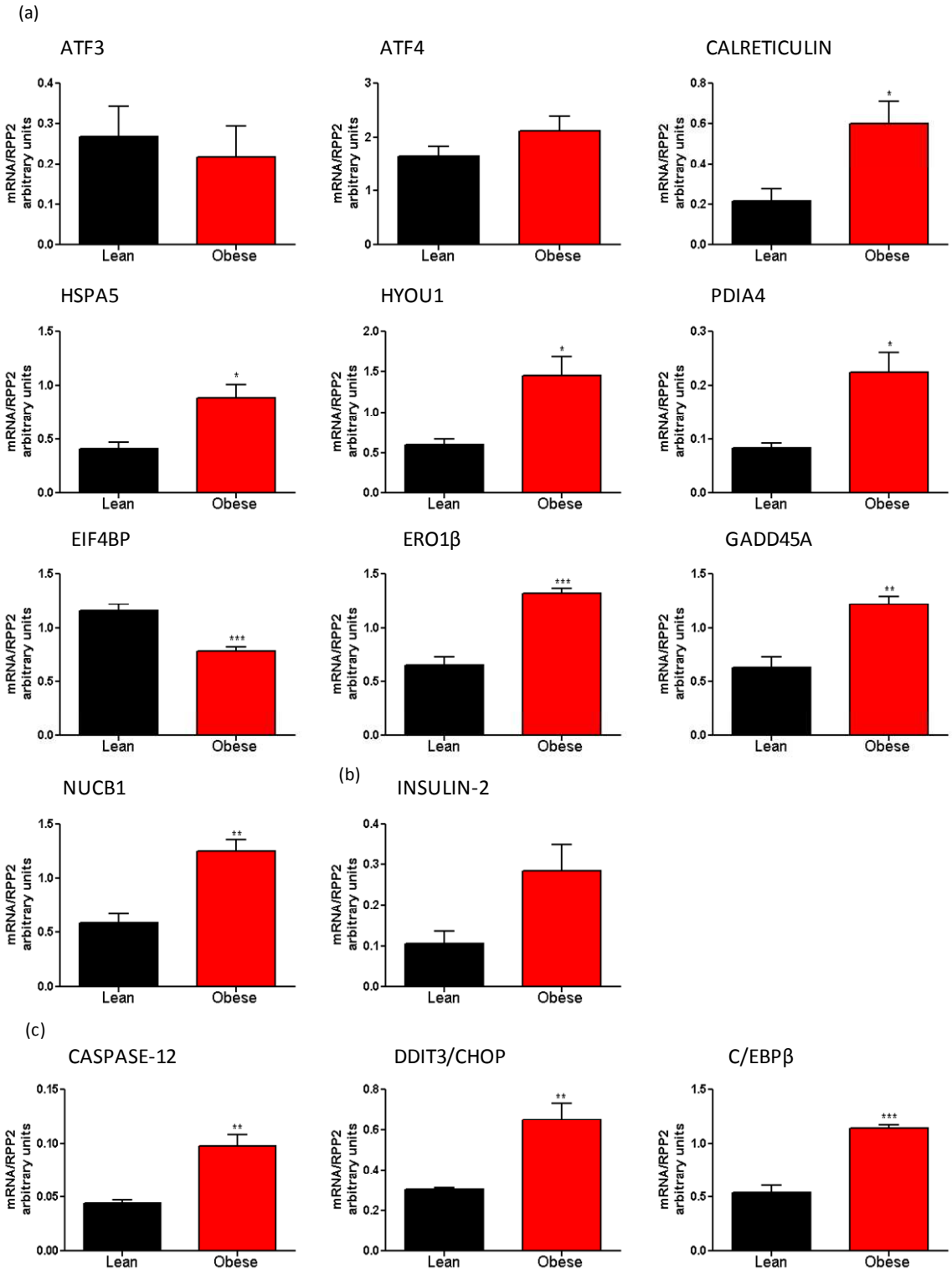


Figure 5.2. Relative expression of select transcripts from islets of obese *vs.* lean Zucker fatty rats by single gene Taqman real time RT-qPCR. Total RNA was extracted from the islets of 11-week old control lean (Fa/Fa) and obese (fa/fa) male Zucker rats and analysed by real-time RT-qPCR for markers of (a) ER stress, (b) β-cell function and (c) apoptosis. Results are expressed as relative expression levels and normalised to the housekeeping gene ribosomal protein P2 (RPP2). Values are mean ± SEM determined from control lean (n=4) and obese rats (n=5). Statistical significance was determined

using an unpaired two-tailed Student's t-test. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$ *vs.* lean control for each gene.

5.2.1.2 Zucker diabetic fatty obese rats

To further investigate whether obesity correlates with the induction of ER stress and the UPR in the islets, changes in the expression of key markers of ER stress and genes encoding markers of β-cell function and differentiation, lipid handling and metabolism and cellular apoptosis were also assessed in 12-week old female ZDFO rats. Female ZDFO rats fed on standard chow diet do not spontaneously develop hyperglycaemia and are characteristically hyperinsulineamic to compensate for insulin resistance and maintain normoglycaemia (Corsetti, Sparks et al. 2000). The 12-week old ZDFO rats weighed significantly more than their age-matched ZDF lean littermates which were used as control rats in this study (**Figure 5.3ai**). In comparison to Zucker rats, the weights of the ZDF rats were observed to be much lower as expected for female ZDF rats in comparison to males. Characteristic hyperinsulineamia with no significant change in fasting blood glucose was observed in the ZDFO rats in comparison to lean controls (**Figure 5.3aii**). A significant increase in plasma triglycerides and glycosylated haemoglobin was also observed in the ZDFO rats in comparison to lean controls (**Figure 5.3aii**).

Islet RNA was extracted from the rats and the comparative expression levels of 44 genes were assessed using islet specific Taqman Low Density Arrays (TLDAs). Statistically significant upregulation in the expression of ER stress markers such as HYOU1, PDIA4, calreticulin, HSPA5, PDIA3 and calnexin was observed in the islets of the ZDFO rats in comparison to lean controls (**Table 5.3**). Although not statistically significant, the expression of Dnajb9 showed a trend towards significant increase (above a 1.5 fold change threshold) in the ZDFO rat islets in comparison to lean controls (**Table 5.3**). The expression of EIF2AK3, ATF4 and MYD116 also showed a trend towards a small increase in the ZDFO rat islets in comparison to lean littermates. This was however not significant (**Table 5.3**).

Analysis of the expression of markers of β-cell function and differentiation showed a general trend towards significant increase in several genes with fold changes above the x1.5 threshold (**Table 5.3**). There was a statistically significant increase in the expression of INS2 and IRS2 genes in the islets of the ZDFO rats in comparison to lean

controls (**Table 5.3**). Statistically significant increase in the expression of SLC2A2/GLUT2 and CCND1 genes was also observed in the islets of the ZDFO rat islets in comparison to lean controls (**Table 5.3**). While a trend towards an increase was observed for PDX1 and GCK in the islets of the ZDFO rats in comparison to lean controls, this change was not significant (**Table 5.3**).

The expression of markers of lipid handling and metabolism was also analysed in the ZDFO rat islets in comparison to ZDF lean controls. A statistically significant increase in the expression of SREBF1, HMGCR and LDLR was observed in the islets of the ZDFO rats in comparison to lean controls (**Table 5.3**). No significant change was observed in the expression of FASN, ACACA and FFAR1/GPR40 encoding genes in the islets of the ZDFO rats in comparison to lean controls (**Table 5.3**).

In correlation with observations made in the islets of the ZO rats, statistically significant increases in the expression of both pro- and anti-apoptotic markers were observed in the islets of the ZDFO rats in comparison to lean controls. Pro-apoptotic genes including BAK1, BAX, BAD and Caspase-12 were all significantly upregulated (**Table 5.3**). The anti-apoptotic BCL2 gene was also significantly upregulated in the islets of the ZDFO rats in comparison to lean controls (**Table 5.3**).

As in the ZO rats, UCP2 was also significantly upregulated in the ZDFO rat islets in comparison to lean ZDF controls (**Table 5.3**).

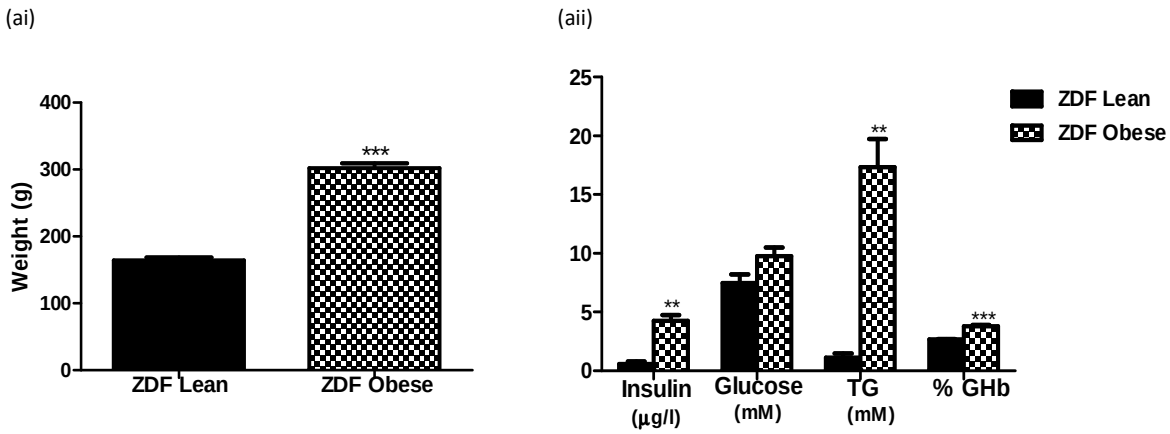


Figure 5.3. Body weights and plasma biochemistry in 12-week old obese *vs.* lean female ZDF rats. (ai) Body weight in *grams* and (aii) plasma insulin, blood glucose, plasma triglyceride and glycosylated haemoglobin levels were measured in obese and lean Zucker rats after an overnight fast. Data are shown as mean ± SEM (ZDFL, n=3; ZDFO, n=3). Statistical significance was determined using an unpaired two-tailed Student's t-test. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$ *vs.* lean control.

Table 5.3

Islet TLDA gene expression results (female ZDF obese rats).

Data obtained for all genes included on islet TLDA and expressed as fold change compared to control female ZDF lean rats on chow diet. Data is normalised to housekeeping gene 18s.

ER stress genes Gene Name	Gene Symbol	ZDFO islets
Hypoxia up-regulated 1	Hyou1	1.77
Wolframin	Wfs1	1.47
DnaJ (Hsp40) homolog, subfamily B, member 9	Dnajb9	1.65
CCAAT/Enhancer binding protein, beta	Cebpb	2.24
Nucleobindin	Nucb1	1.92
Protein disulphide isomerase family A, member 3	Pdia3	1.55
Paraoxonase 2	Pon2	1.69
Protein disulphide isomerase family A, member 4	Pdia4	2.06
Calnexin	Canx	1.54
UDP-glucose ceramide glucosyltransferase-like 1	Ugcgl1	1.42
Calreticulin	Calr	1.87
Growth arrest and DNA-damage-inducible, alpha	Gadd45a	2.22
Activating transcription factor 5	Atf5	-1.19
Mitogen-activated protein kinase 8	Mapk8	1.62
PRKR-like endoplasmic reticulum kinase	Eif2ak3	1.26
Heat shock 70 kDa protein 5	Hspa5	1.84
Growth arrest and DNA-damage-inducible protein	Ddit3	1.55
Myeloid differentiation primary response gene 116	Myd116	1.17
DnaJ (Hsp40) homolog, subfamily C, member 3	Dnajc3	1.23
Activating transcription factor 4	Atf4	1.24
Homocysteine-inducible, ER stress-inducible	Herpud1	1.31
Tribbles 3	Trib3	1.35
X-box binding protein 1	Xbp1	1.00
Quiescin Q6 sulfhydryl oxidase 1	Qscn6	-1.04
FK506 binding protein 11	Fkbp11	1.05
Activating transcription factor 3	Atf3	-1.39

β-cell function & differentiation genes Gene Name	Gene Symbol	ZDFO islets
Insulin 2	Ins2	2.36
Cyclin D1	Ccnd1	3.82
Glucose transporter type 2	Slc2a2/Glut2	2.20
Pancreatic and duodenal homeobox 1	Pdx1	1.38
Insulin Receptor Substrate 2	Irs2	2.04
Glucokinase/ Hexokinase 4	Gck	1.38

Lipid handling and metabolism genes Gene Name	Gene Symbol	ZDFO islets
Sterol Regulatory Element Binding Protein–1	Srebf1	1.31
HMG-CoA reductase	Hmgcr	2.91
Low density lipoprotein receptor	Ldlr	1.95
Fatty Acid Synthase	Fasn	-1.00
Acetyl CoA Carboxylase	Acaca	1.21
Free fatty acid receptor 1	Ffar1	-1.20

Apoptosis genes Gene Name	Gene Symbol	ZDFO islets
Bcl-2 homologous antagonist/killer	Bak1	2.44
B-cell lymphoma protein 2	Bcl2	1.82
Caspase 12	Casp 12	2.85
BCL2-associated X protein	Bax	1.73
BCL-X/BCL-2 binding protein	Bad	1.65

Miscellaneous genes Gene Name	Gene Symbol	ZDFO islets
Uncoupling protein 2	Ucp2	2.63

5.2.1.2.1 Transcriptional analysis by single gene Taqman RT-qPCR

To confirm the gene expression data obtained using the TLDAs, the expression of a selected set of genes was further analysed by single gene quantitative RT-PCR. Genes encoding ER stress markers such as calreticulin, HSPA5 and PDIA4 were significantly upregulated in the islets of the ZDFO rats in comparison to lean controls (**Figure 5.4a**). Although not statistically significant, the expression of HYOU1 and GADD45A showed a trend towards an increase in the islets of the ZDFO rats in comparison to ZDFL rat islets (**Figure 5.4a**). While NUCB1 was also significantly upregulated, the expression levels of ERO1β, ATF3 and ATF4 were unchanged in the ZDFO rat islets in comparison to lean control rat islets (**Figure 5.4a**). Similar to the observation in the ZO rat islets, the expression of EIF4BP was significantly downregulated in the ZDFO rat islets in comparison to lean control (**Figure 5.4a**). Although not statistically significant, there was a trend towards an increase in the expression of the insulin (INS2) gene in the islets of the ZDFO rats in comparison to lean controls indicative of a mild increase in β-cell function (**Figure 5.4b**). Assessment of the expression levels of apoptotic markers showed a statistically significant increase in the expression of caspase-12 in the islets of the ZDFO rats in comparison to lean controls (**Figure 5.4c**). Although not statistically significant, there was a trend towards an increase in the expression of DDIT3/CHOP and C/EBPβ in the ZDFO rat islets in comparison to lean controls (**Figure 5.4c**).

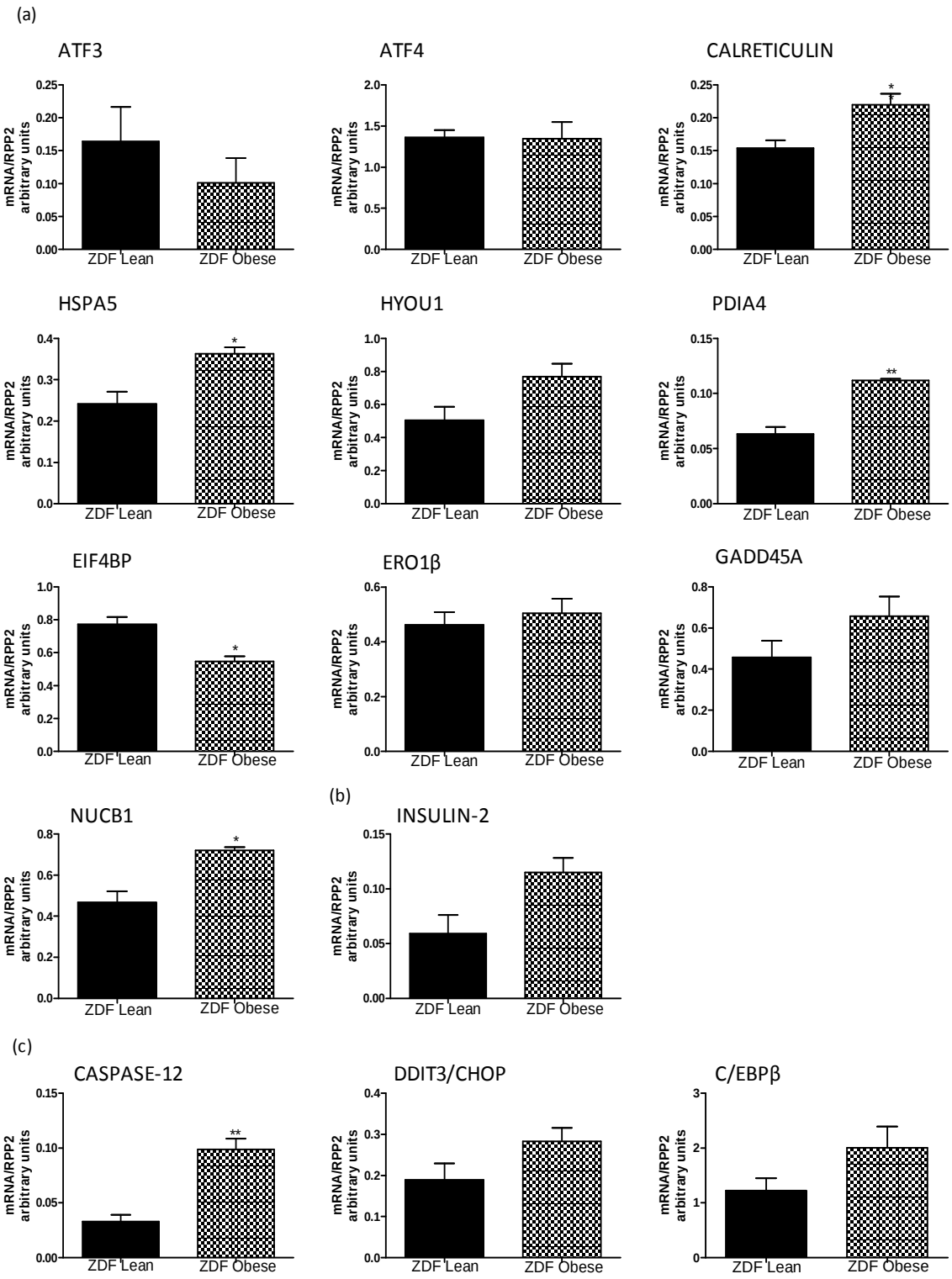


Figure 5.4. Relative expression of select transcripts from islets of obese *vs.* lean ZDF rats by single gene Taqman real time RT-qPCR. Total RNA was extracted from the islets of 12-week old control lean (Fa/Fa) and obese (fa/fa) female ZDF rats and analysed by real-time RT-qPCR for markers of (a) ER stress, (b) β-cell function and (c) apoptosis. Results are expressed as relative expression levels and normalised to the housekeeping gene ribosomal protein P2 (RPP2). Values are mean ± SEM determined from control lean (n=3) and obese rats (n=3). Statistical significance was determined

using an unpaired two-tailed Student's t-test. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$ *vs.* lean control for each gene.

5.2.2 ER stress is not significantly induced in the diet induced murine model of obesity

To further determine the induction of ER stress and the UPR under conditions of obesity, changes in the expression of key markers of ER stress, β-cell function and apoptosis were also assessed in the islets of diet induced obese (DIO) mice in comparison to their lean littermates. The DIO mice in this study were generated by placing 7 week old C57B16Jax mice on a 40 kcal% fat rodent diet (high fat diet) (C13004i Research Diets, USA) for 31 weeks. The DIO mice weighed significantly more than the age matched lean control mice fed on a standard chow diet over the same period (**Figure 5.5ai**). Diet induced obesity in the mice also resulted in characteristic hyperinsulineamia but had no effect on blood glucose levels in comparison to lean control mice (**Figure 5.5aii**).

Single gene quantitative real time RT-PCR was used to analyse the expression of a selected number of genes in the islets of the DIO mice in comparison to lean control mice fed on a standard chow diet. Although the changes in gene expression were not statistically significant, the expression of genes encoding chaperone proteins and foldases such as calreticulin, HSPA5, HYOU1 and PDIA4 showed a trend towards an increase in the islets of the DIO mice in comparison to lean controls (**Figure 5.5bi**). The expression of ATF4 was unchanged while there was a trend towards a decrease in the expression of ATF3 in the DIO mice islets in comparison to lean controls (**Figure 5.5bi**). Despite significant hyperinsulineamia in the blood plasma, there was no detectable change in the expression of the insulin (INS2) gene in the DIO mice islets in comparison to lean control islets (**Figure 5.5bii**). The expression of the apoptotic markers DDIT3/CHOP and caspase-12 was also assessed. No significant change was observed in the expression of CHOP and caspase-12 in the islets of the DIO mice in comparison to lean control mice islets (**Figure 5.5biii**).

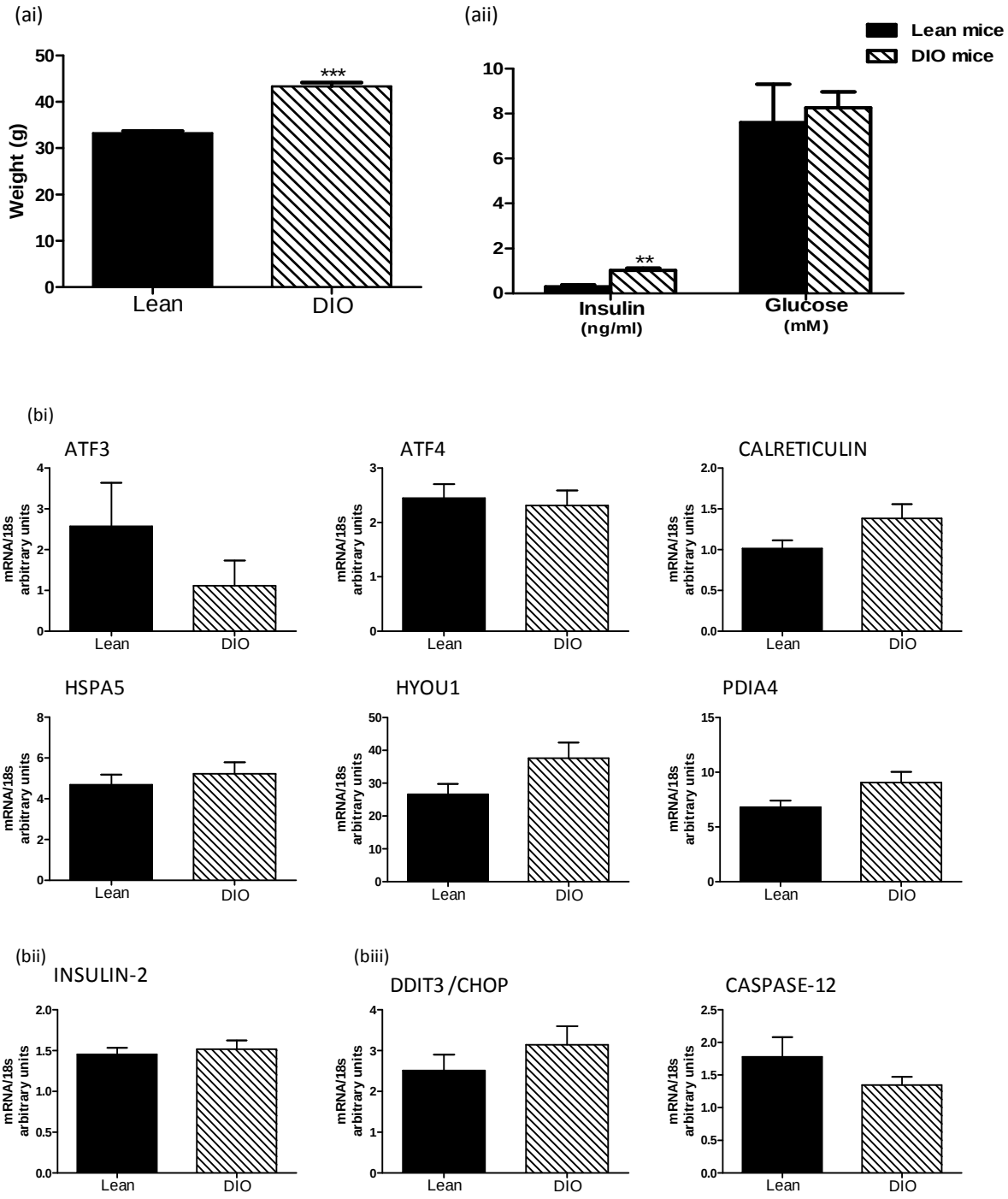


Figure 5.5. Body weight, plasma biochemistry and relative expression of select transcripts from islets of 38-week old lean and DIO male C57BL/6J mice. (ai) Body weight in *grams* and (aii) plasma insulin and blood glucose were measured in lean and DIO mice after a 16h overnight fast. Total RNA was extracted from the islets and analysed by Taqman real-time RT-qPCR for markers of (bi) ER stress, (bii) β-cell function and (biii) apoptosis. Results are expressed as relative expression levels and normalised to the housekeeping gene ribosomal r18s. Values are mean ± SEM determined from control lean (n=8) and DIO mice (n=12). Statistical significance was determined using an unpaired two-tailed Student's t-test. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$ *vs.* lean control for each gene.

5.2.3 Induction of diabetes in female ZDFO rats does not correlate with an increase in the expression of ER stress markers

Data presented here and previously published by others (Huang, Lin et al. 2007, Matveyenko, Gurlo et al. 2009) demonstrate that obesity induces an increase in the expression of markers of ER stress in the islets. The chronic induction of ER stress has been suggested as a mechanism by which β-cell dysfunction and apoptosis is induced in type 2 diabetes (Cnop, Welsh et al. 2005). To investigate the correlation between the development of hyperglycaemia and ER stress signalling, female ZDFO rats were placed on a high fat diet for 7 weeks to induce β-cell dysfunction and diabetes (Corsetti, Sparks et al. 2000). High fat fed ZDFO rats weighed significantly more than their obese littermates fed on a standard chow diet for the same duration (**Figure 5.6ai**). A significant decline in fasting plasma insulin was indicative of severe β-cell dysfunction in the ZDFO-HFD rats in comparison to chow fed ZDFO rats (**Figure 5.6aii**). This significant decrease in circulating insulin levels correlated with equally significant fasting hyperglycaemia in the ZDFO-HFD rats in comparison to chow fed obese controls (**Figure 5.6aii**).

Islet RNA was extracted from the rats and the comparative expression levels of 44 genes were assessed using islet specific Taqman Low Density Arrays (TLDAs). In contrast to euglycaemic chow fed ZDFO rats, there was a general trend towards a decrease in the expression of ER stress markers in the islets of the ZDFO-HFD rats (**Table 5.4**). Chaperone proteins and foldases, such as HYOU1, DNAJB9, PDIA4, calreticulin, and HSPA5 were all significantly down-regulated beyond the x1.5 fold threshold in the ZDFO-HFD rat islets in comparison to chow fed ZDFO control islets (**Table 5.4**). Statistically significant downregulation was also observed in the expression of Wfs1, calnexin and EIF2AK3 in the ZDFO-HFD rats islets in comparison to chow fed ZDFO control islets (**Table 5.4**). While the expression of PERK downstream effectors such as ATF4 and MYD116 genes remained unchanged, ATF3 showed a significant decline beyond the 1.5 fold change threshold in the ZDFO-HFD rat islets in comparison to chow fed ZDFO controls (**Table 5.4**).

Markers of β-cell function and differentiation were also significantly down-regulated in the islets of the ZDFO-HFD rats in comparison to chow fed control ZDFO rat islets. A statistically significant decrease in the expression of the insulin gene (INS2), SLC2A2/GLUT2 and GCK genes and a significant (over x1.5 threshold) downregulation of IRS2 is indicative of a decline in β-cell function in the islets of the ZDFO-HFD rats in comparison to chow fed ZDFO controls (**Table 5.4**). In addition, statistically significant downregulation of PDX1 coupled with the significant (over x1.5 threshold) downregulation of CCND1 was also observed in the islets of the ZDFO-HFD rats in comparison to chow fed control ZDFO rats (**Table 5.4**). This is indicative of a decline in the replication machinery and perhaps, an inability to compensate for insulin resistance through an increase in β-cell mass in the ZDFO-HFD rats in comparison to chow fed ZDFO control rats.

A general trend towards downregulation was also observed in the expression of lipid handling and metabolism markers (**Table 5.4**). While the downregulation of SREBF1 and LDLR were statistically significant, significant (over x1.5 threshold) downregulation was also observed for Hmgcr, FASN, and FFAR1/GPR40 genes in the islets of the ZDFO-HFD rats in comparison to chow fed ZDFO rat islets (**Table 5.4**). The expression of the ACACA gene remained unchanged in the islets of the ZDFO-HFD rats in comparison to chow fed ZDFO islet controls (**Table 5.4**).

Surprisingly, both pro- and anti-apoptotic markers were significantly downregulated in the islets of the ZDFO-HFD rats in comparison to controls (**Table 5.4**). Statistically significant decline was observed in the expression of pro-apoptotic genes such as BAK1, BAD, BAX and caspase-12. Statistically significant downregulation in the expression of the anti-apoptotic BCL2 gene was also observed (**Table 5.4**).

UCP2 is reportedly upregulated as a result of exposure to FFAs (Lameloise, Muzzin et al. 2001). In the islets of the ZDFO-HFD rats however, there was a statistically significant decline in the expression of UCP2 in comparison to expression levels in the islets of chow fed ZDFO control rats (**Table 5.4**).

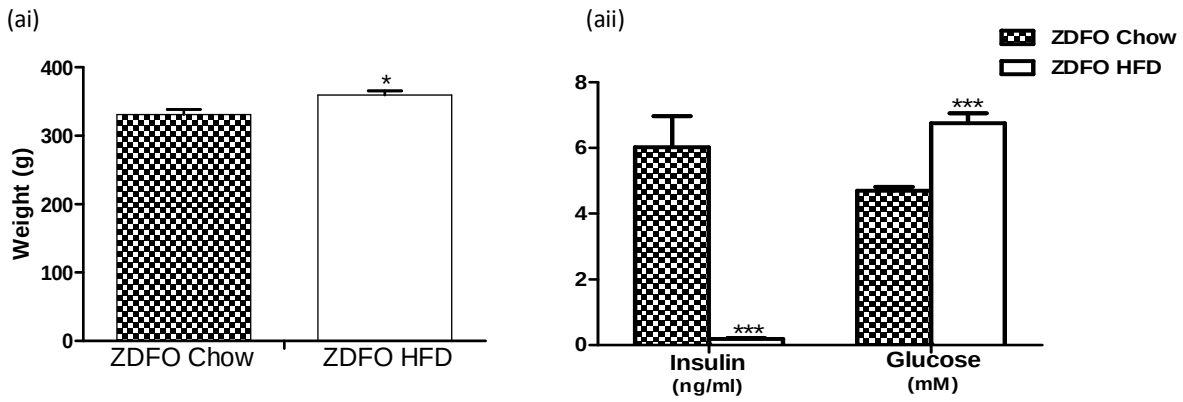


Figure 5.6. Body weights and plasma biochemistry in 12-week old high fat diet fed ZDFO (ZDFO HFD) *vs.* chow fed ZDFO rats. (ai) Body weight in *grams* and (aii) plasma insulin and blood glucose levels were measured in chow fed ZDFO and high fat fed ZDFO rats after an overnight fast. Data are shown as mean ± SEM (ZDFO chow, n=6; ZDFO HFD, n=6). Statistical significance was determined using an unpaired two-tailed Student's t-test. *, *P*<0.05; **, *P*<0.01; ***, *P*<0.001 *vs.* chow fed obese control.

Table 5.4

Islet TLDA gene expression results (female ZDFO-HFD rats).

Data obtained for all genes included on islet TLDA and expressed as fold change compared to control female ZDF obese rats on chow diet. Data is normalised to housekeeping gene 18s.

ER stress genes Gene Name	Gene Symbol	ZDFOD islets
Hypoxia up-regulated 1	Hyou1	-1.51
Wolframin	Wfs1	-1.72
DnaJ (Hsp40) homolog, subfamily B, member 9	Dnajb9	-1.64
CCAAT/Enhancer binding protein, beta	Cebpb	-2.06
Nucleobindin	Nucb1	-1.87
Protein disulphide isomerase family A, member 3	Pdia3	-1.33
Paraoxonase 2	Pon2	-1.24
Protein disulphide isomerase family A, member 4	Pdia4	-1.62
Calnexin	Canx	-1.45
UDP-glucose ceramide glucosyltransferase-like 1	Ugcgl1	-1.49
Calreticulin	Calr	-1.51
Growth arrest and DNA-damage-inducible, alpha	Gadd45a	-1.59
Activating transcription factor 5	Atf5	1.33
Mitogen-activated protein kinase 8	Mapk8	-1.80
PRKR-like endoplasmic reticulum kinase	Eif2ak3	-1.48
Heat shock 70 kDa protein 5	Hspa5	-1.61
Growth arrest and DNA-damage-inducible protein	Ddit3	-1.81
Myeloid differentiation primary response gene 116	Myd116	-1.47
DnaJ (Hsp40) homolog, subfamily C, member 3	Dnajc3	1.00
Activating transcription factor 4	Atf4	-1.23
Homocysteine-inducible, ER stress-inducible	Herpud1	1.37
Tribbles 3	Trib3	1.22
X-box binding protein 1	Xbp1	-1.32
Quiescin Q6 sulfhydryl oxidase 1	Qscn6	-1.24
FK506 binding protein 11	Fkbp11	-1.03
Activating transcription factor 3	Atf3	-1.72

β-cell function & differentiation genes Gene Name	Gene Symbol	ZDFOD islets
Insulin 2	Ins2	-2.38
Cyclin D1	Ccnd1	-2.95
Glucose transporter type 2	Slc2a2/Glut2	-2.35
Pancreatic and duodenal homeobox 1	Pdx1	-2.12
Insulin Receptor Substrate 2	Irs2	-1.89
Glucokinase/ Hexokinase 4	Gck	-2.3

Lipid handling and metabolism genes Gene Name	Gene Symbol	ZDFOD islets
Sterol Regulatory Element Binding Protein–1	Srebf1	-1.68
HMG-CoA reductase	Hmgcr	-2.52
Low density lipoprotein receptor	Ldlr	-2.6
Fatty Acid Synthase	Fasn	-1.58
Acetyl CoA Carboxylase	Acaca	-1.15
Free fatty acid receptor 1	Ffar1	-1.85

Apoptosis genes Gene Name	Gene Symbol	ZDFOD islets
Bcl-2 homologous antagonist/killer	Bak1	-2.3
B-cell lymphoma protein 2	Bcl2	-1.76
Caspase 12	Casp 12	-2.71
BCL2-associated X protein	Bax	-2.2
BCL-X/BCL-2 binding protein	Bad	-1.74

MIscellaeous genes Gene Name	Gene Symbol	ZDFOD islets
Uncoupling protein 2	Ucp2	-2.28

5.2.3.1 Transcriptional analysis by single gene Taqman RT-qPCR

To confirm the gene expression data obtained using the TLDAs, the expression of a selected set of genes was further analysed by single gene real time quantitative RT-PCR. The expression levels of ER stress markers such as calreticulin, HSPA5, HYOU1 and PDIA4 were unchanged in the ZDFO-HFD islets in comparison to chow fed ZDFO control islets (**Figure 5.7a**). The expression of other ER stress markers such as ATF3, ATF4, EIF4BP, ERO1β, GADD45A and NUCB1 also remained unchanged in the islets of the ZDFO-HFD rats in comparison to chow fed ZDFO control rat islets (**Figure 5.7a**). The expression of the insulin-2 gene was also assessed as a marker of β-cell function. No significant change was observed in the expression of insulin-2 in the islets of the ZDFO-HFD rats in comparison to chow fed ZDFO control rats (**Figure 5.7b**). The assessment of the expression levels of apoptotic markers also showed no significant change in the expression of caspase-12, DDIT3/CHOP and C/EBPβ in the islets of the ZDFO-HFD rats in comparison to chow fed ZDFO control rat islets (**Figure 5.7c**). These data suggest that despite the apparent development of β-cell dysfunction, ER stress in the islets of the chow fed ZDFO rats is not potentiated in the diabetic high fat fed ZDFO rat islets.

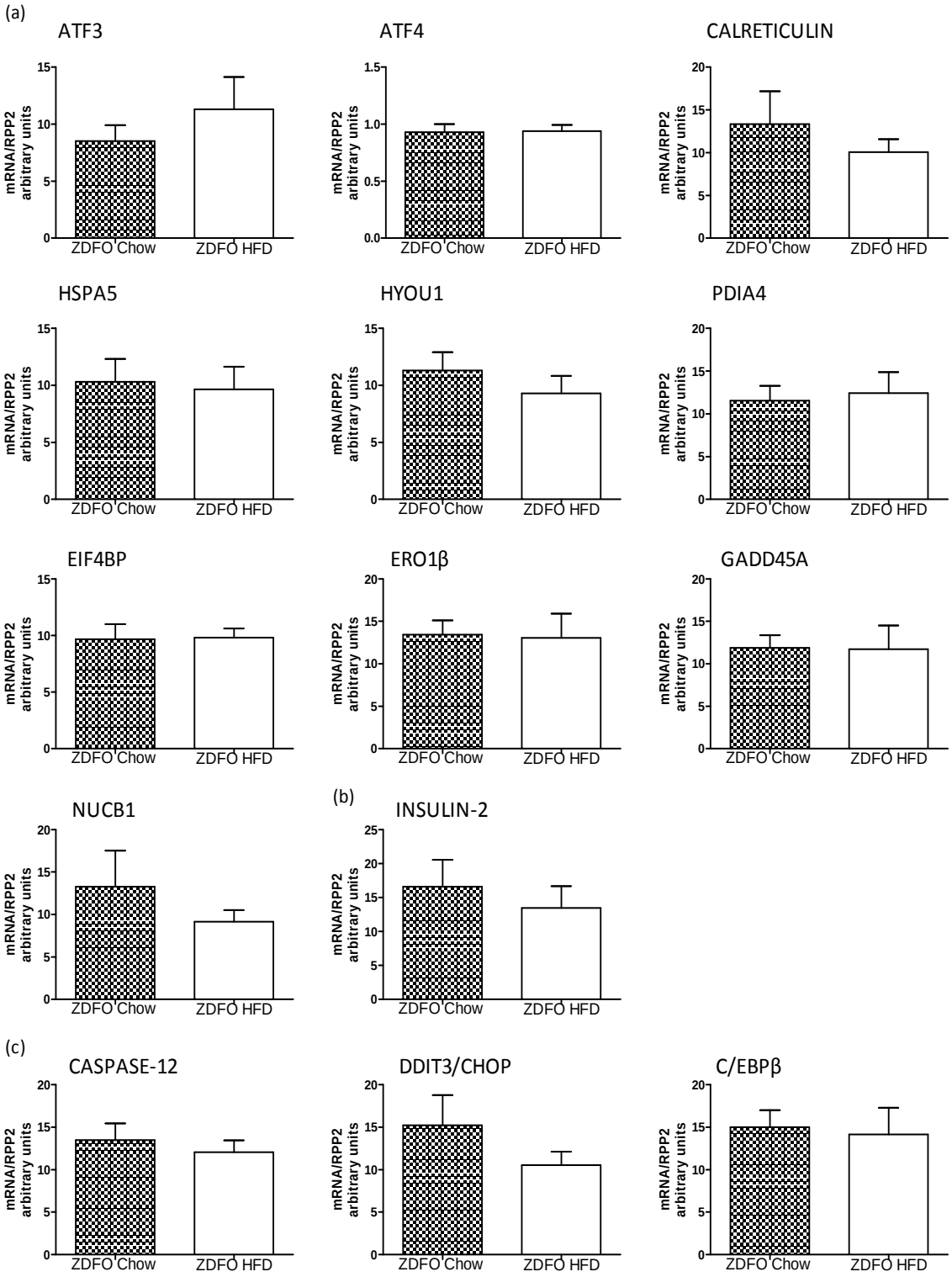


Figure 5.7. Relative expression of select transcripts from islets of ZDFO HFD *vs.* chow fed ZDFO rats by single gene Taqman RT-qPCR. Total RNA was extracted from the islets of 12-week old control chow fed (fa/fa) and high fat fed (fa/fa) female ZDFO rats and analysed by real-time RT-qPCR for markers of (a) ER stress, (b) β-cell function and (c) apoptosis. Results are expressed as relative expression levels and normalised to the housekeeping gene ribosomal protein P2 (RPP2). Values are mean ± SEM determined from control chow fed ZDFO (n=6) and ZDFO HFD rats (n=6). Statistical significance was

determined using an unpaired two-tailed Student's t-test. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$ *vs.* chow fed obese control for each gene.

5.2.4 Upregulation of ER stress markers in female ZDF obese rats is not potentiated in diabetic female ZDF obese rat islets

Analysis of the transcriptional profiles of ER stress markers in obese ZDF rats showed an increase which was not potentiated when diabetes was induced. In fact, there was no potentiation of any responses in the diabetic ZDFO-HFD rat islets in comparison to ZDFO rats on the chow diet. Changes in mRNA expression however, do not necessarily reflect changes in protein expression. The protein expression profile of ER stress markers was therefore determined in the islets of chow fed lean (ZDFL), chow fed obese (ZDFO) and high fat fed obese (ZDFO-HFD) ZDF rats by Western blotting. Increased protein expression was observed for calreticulin, HSPA5 and HYOU1 in the islets of the ZDFO rats in comparison to expression levels in lean control ZDF rat islets (**Figure 5.8a**), correlating with the observed increase in transcription (**Figure 5.4a**). However, there was no potentiation in the expression of these proteins in the islets when β-cell dysfunction and diabetes was induced in the ZDFO HFD rats in comparison to chow fed ZDFO rat islets (**Figure 5.8a**). The protein expression level of 4EBP1 was also assessed in the rat islets. While the transcriptional expression of EIF4BP was downregulated in the islets of the chow fed ZDFO rats, there was a trend towards an increase in the expression of the protein when assessed by Western blotting (**Figure 5.8a**). No further increase in the expression of 4EBP1 was observed in the islets of the ZDFO-HFD rats in comparison to chow fed ZDFO rat islets. While the phosphorylation state of IRE1α and the protein levels of spliced XBP1 was undetectable in the rodent islets, unspliced XBP1 protein expression was detected. The activation of IRE1 results in an increase in the levels of spliced XBP1 from a pool of total XBP1 in the cell. Therefore by inference, an increase in the levels of unspliced XBP1 is indicative of a decrease in the levels of the spliced form of XBP1. There was a decrease in the expression of unspliced XBP1 in the islets of the chow fed ZDFO rats in comparison to lean ZDF rat islets, indicative of the activation of IRE1 function (**Figure 5.8a**). The levels of unspliced XBP1 however was unchanged between the islets of the chow fed ZDFO and the ZDFO-HFD rats, suggesting that the activation of IRE1 is not potentiated in the islets of the ZDFO-HFD rats (**Figure 5.8a**). The expression of phosphorylated JNK in the rat islets was also detected. JNK phosphorylation may be indicative of the chronic

activation of IRE1 signalling (Urano, Wang et al. 2000) or as a marker of impaired insulin signalling in the islets (Cunha, Hekerman et al. 2008, Aguirre, Uchida et al. 2000). A significant increase in the phosphorylation state of JNK was observed in the islets of the chow fed ZDFO rats in comparison to lean control rat islets (**Figure 5.8b**). No statistically significant increase in the phosphorylation of JNK was observed in the islets of the ZDFO HFD rats in comparison to chow fed ZDFO rat islets (**Figure 5.8b**). JNK phosphorylation in sorbitol treated MIN6 cell and Wistar rat islets are shown as controls. Sorbitol was used as a specific inducer of JNK phosphorylation (Ip, Davis 1998). The expression of the pro-apoptotic CHOP was also assessed in the islets of the chow fed ZDFO and ZDFO HFD rats in comparison to lean control rat islets. An increase in the expression of CHOP was observed in the islets of the chow fed ZDFO rats in comparison to lean control rat islets with no further potentiation in the islets of the ZDFO HFD rats (**Figure 5.8a**). The expression levels of GAPDH and ribosomal protein S6 were also detected as protein loading controls.

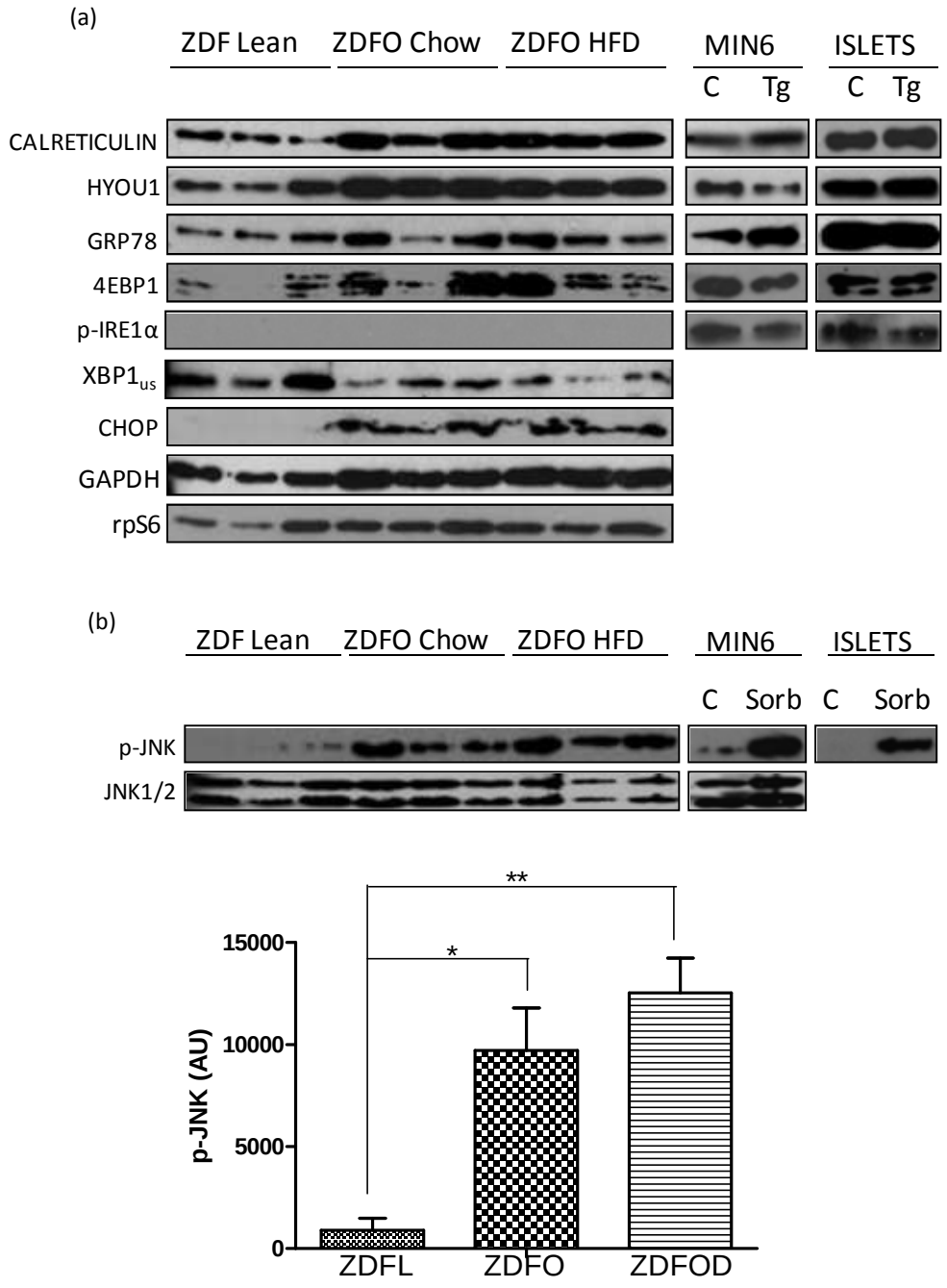


Figure 5.8. Expression of downstream markers of the UPR in pancreatic islets of chow fed ZDFL, chow fed ZDFO and high fat fed ZDFO rats. Islets were isolated from the pancreas and suspended in 1X Laemmli sample buffer. Proteins were resolved on SDS-PAGE and Western blotted using antisera against (a) calreticulin, 4EBP1, Hyou1, Grp78, p-IRE1α, XBP1, and CHOP. GAPDH and rpS6 were detected as protein loading controls. Samples were also Western blotted using antisera against (b) p-JNK and total JNK1/2 as a loading control. MIN6 cells and rats islets were either treated with 1μM thapsigargin for 2h (MIN6) or 18h (islets) or 1M sorbitol for 0.5h. Samples were lysed and western blotted as antibody controls. Western blots were quantified using ImageJ software. Protein expression in islets and MIN6 cells are shown as antibody controls. Blots shown are representative of samples from 3 different animals in each group.

5.3 Discussion

The data presented in this study confirm previously published reports on the adaptation of β-cell function and mass to compensate for insulin resistance in obesity. This study also provides evidence which suggests the induction of an adaptive ER stress response in the islets of genetic rodent models of obesity in comparison to appropriate controls. In addition, evidence presented in this study suggests that the development of β-cell dysfunction in obesity associated type 2 diabetes occurs independently of chronic ER stress signalling. The induction of adaptive ER stress in obesity and the lack of potentiation in the development of type 2 diabetes therefore indicate that ER stress may only play an adaptive role in the pathogenesis of type 2 diabetes.

5.3.1 Obesity induces an adaptive increase in β-cell function

Normal compensation for obesity induced insulin resistance involves both the altered regulation of metabolic enzymes which control β-cell sensitivity to glucose and increases in β-cell mass (Milburn, Hirose et al. 1995, Cockburn, Ostrega et al. 1997). Although β-cell mass was not directly assessed in this study, several studies have previously reported significant increase in β-cell mass in rodent models of obesity (Flier, Kulkarni et al. 2001, Matveyenko, Gurlo et al. 2009, Milburn, Hirose et al. 1995) and human obese subjects in comparison to lean controls (Ogilvie 1933, Butler, Janson et al. 2003a). The significant increase in the expression of an important cell proliferation marker, cyclin D1 (CCND1) and the concomitant increase in PDX1 in the islets of the obese rats in this study suggest the stimulation of β-cell growth and β-cell differentiation in comparison to lean controls.

Significant upregulation of genes involved in maintaining β-cell function and differentiation was indicative of islet functional adaptation to obesity in the ZO and ZDFO rats. The expression of GLUT2/SLC2A2, the major glucose transporter involved in islet glucose uptake, and glucokinase (GCK) are strongly and positively linked to the differentiation state of the β-cells, and are both under the regulatory control of the pancreatic duodenal homeobox-1 (PDX1) transcription factor (Lebrun, Montminy et al. 2005). Upregulation of GLUT2 and GCK is suggestive of an attempt to increase

glycolytic flux thereby lowering blood glucose to maintain normoglycaemia. This observation is supported by a study in which total glucose utilisation was found to be increased by x1.5-2 fold in ZO rats in comparison to lean controls (Liu, Jetton et al. 2002). In addition to its role in the regulation of glucose metabolism, a recent study has provided evidence suggesting a functional role for GCK activity in the proliferation of β-cells (Porat, Weinberg-Corem et al. 2011). An increase in GCK expression in the islets of the ZO and ZDFO rats in comparison to lean controls is therefore suggestive of an increase in β-cell proliferation in response to obesity. In addition to the regulation of GLUT2 and GCK expression, PDX1 is important for the maintenance of β-cell differentiation, thus providing a link between the factors and pathways required for both β-cell growth and β-cell function.

In addition to the upregulation of metabolic enzymes, insulin resistance requires a concomitant increase in insulin biosynthesis and secretion to maintain enhanced β-cell function (Melloul, Marshak et al. 2002). Indeed, the obese rats in this study were significantly hyperinsulineamic with a trend towards an increase in the mRNA expression of the insulin (INS2) and IRS2 genes, indicative of an increase in the β-cell capacity for insulin synthesis and secretion. Hence, the obesity induced increase in the expression of markers of β-cell function and differentiation likely contributes to compensatory hyperinsulineamia and an increase in the signalling pathways involved in insulin biosynthesis and secretion and glucose metabolism to maintain normoglycaemia in the obese rat models.

In addition to an increase in glycolytic flux, compensatory increase in β-cell function in response to obesity and insulin resistance may also be stimulated through an increase in lipid signalling. Although the mechanisms have not been fully elucidated, it is well established that a supply of FFAs is essential for normal GSIS (Dobbins, Chester et al. 1998) and the amplification of GSIS (Prentki, Joly et al. 2002). Several markers of lipid handling and metabolism were found to be upregulated in the islets of the obese rats in comparison to their lean littermates. In the islets of the male ZO but not the female ZDFO rats, the expression of the gene encoding a FFA receptor FFAR1/GPR40 was found to be significantly upregulated in comparison to lean controls. Binding of FFAs to GPR40 results in the activation of an intracellular signalling cascade which culminates

in an increase in intracellular Ca^{2+} levels and the stimulation of insulin secretion (Itoh, Kawamata et al. 2003, Steneberg, Rubins et al. 2005). Upregulation of GPR40 expression in the islets of the ZO rats may therefore contribute to an increase in insulin secretion from the β-cell. It is unclear why the FFAR1 gene was not upregulated in the islets of the female ZDFO rats. Indeed, changes in gene expression were found to be more pronounced in the male ZO rat islets than in the female ZDFO rat islets in comparison to appropriate controls. Although the reason for this is unclear, it may perhaps be attributed to the gender difference in the animals. Characterisation of triglyceride content in both male and female ZDFO rats revealed that females, whether diabetic or not, have triglyceride-rich lipoprotein particles which are more triglyceride-enriched than corresponding particles in males (Corsetti, Sparks et al. 2000). This was consistent with findings in human studies which showed smaller low density lipoprotein (LDL) particles which are derived from very low density lipoprotein (VLDL) in women than in men with non-insulin dependent diabetes mellitus (Haffner, Mykkanen et al. 1994, Musliner, McVicker et al. 1987). Sex hormones such as oestrogen have been suggested to improve lipid homeostasis and confer more resistance to the development of insulin resistance in females than in males through signalling events downstream of the estrogen receptors and a membrane bound GPCR for oestrogen known as GPR30 (Kuhl, Hilding et al. 2005, Macotela, Boucher et al. 2009, Tiano, Delghingaro-Augusto et al. 2011). As such, resistance to the development of insulin resistance in the female ZDFO rats in comparison to the male ZO rats may translate into the reduced effects in gene expression observed in this study.

The expression of other markers of lipid handling and metabolism such as Srebf1, HMGCR and LDLR were also significantly upregulated in the islets of the obese rats in comparison to lean controls. These markers of lipogenesis have been suggested to play physiological roles in the pancreatic β-cell. In particular, SREBP1 mediated increase in lipid synthesis has been suggested to be required for the adaptive changes in islet gene expression and insulin secretion that occur at high glucose concentrations (Diraison, Ravier et al. 2008). In agreement with the observed increase in the SREBF1 gene in the islets of the obese rats in this study, an increase in the SREBP1 gene was also reported in a study in normoglycaemic but insulin resistant *ob/ob* mice in

comparison to wild type C57BL/6 control mice (Medina-Gomez, Yetukuri et al. 2009). In this regard, an increase in SREBF1 might represent an adaptation to promote lipogenesis in response to changes in glucose levels and thus stimulate the effect of fatty acids on insulin secretion.

5.3.2 Obesity induces the activation of an adaptive UPR

In addition to compensatory increases in β-cell function, the induction of an adaptive UPR was also observed in the islets of the obese rats in comparison to lean controls. Although ER stress induction has previously been reported in the islets of some human subjects and animal models of obesity associated diabetes (Laybutt, Preston et al. 2007, Marchetti, Bugliani et al. 2007), this study reports for the first time, the induction of obesity induced ER stress in the islets of the ZO and ZDFO rats in the absence of diabetes. Both ZO and ZDFO rats displayed significant upregulation in the mRNA expression of key ER stress markers such as HYOU1, HSPA5/BiP, PDIA4, calreticulin, calnexin and nucleobindin in their islets in comparison to their lean littermates. This is indicative of the induction of an adaptive UPR to increase protein folding capacity within the ER. This result was confirmed by the increase in protein expression of HSPA5/BiP, HYOU1 and calreticulin in the islets of the ZDFO rats in comparison to lean controls as assessed by Western blotting. Upregulation of chaperone protein expression under ER stress conditions is induced by the activity of all three transducer proteins present on the membrane of the ER. It was apparent in this study however, that the PERK pathway was not activated in the islets of the obese rats as the upregulation of the PERK encoding gene (EIF2AK3) in the islets of the ZO rats did not translate into the downstream upregulation of its downstream target genes ATF4 and ATF3. Indeed, the expression of ATF4 and ATF3 remained unchanged in the islets of the obese rats in comparison to lean controls. Furthermore, the expression of the EIF4BP gene which is dependent on ATF4 expression (Suguru Yamaguchi, Hisamitsu Ishihara et al. 2008), was found to be significantly downregulated in the islets of both obese rat models in comparison to lean controls, indicating the lack of ATF4 activity in the obese rat islets. Although the expression of ATF4 mRNA was not altered in the islets of the obese rats, ATF4 expression was significantly upregulated in the adipose tissue of the ZO rats in comparison to lean

controls, an indicator that the assay was efficient for the detection of this gene (**Appendix 1.1**). The lack of upregulation of PERK target genes in the islets of the obese rats suggests that the increase in chaperone protein expression observed was mediated by the activity of IRE1 and/or ATF6. While IRE1 gene expression was not assessed in this study and no significant change was observed in the mRNA expression of total XBP1, a decrease in the protein expression of unspliced XBP1 was observed in the islets of the ZDFO rats in comparison to lean controls. This suggests that IRE1 may have been activated in the islets of the obese rats in comparison to lean controls.

Induction of chronic ER stress in response to elevated FFA levels has been implicated in the development of β-cell dysfunction and apoptosis in obesity associated type 2 diabetes (Prentki & Nolan 2006). Chronic exposure of clonal β-cell lines and rat islets to saturated FFAs has been shown to induce apoptosis in several studies (Kharroubi, Ladriere et al. 2004, Cunha, Hekerman et al. 2008, Karaskov, Scott et al. 2006). An increase in the expression of DDIT3/CHOP was also reported in the islets of obese non-diabetic human subjects in comparison to lean non-diabetic controls in a study by Huang et al., (Huang, Lin et al. 2007). In correlation with these studies, the expression of apoptosis markers such as CHOP and caspase-12 was upregulated in the islets of the obese rats in comparison to lean controls. There was however, no evidence of dysfunction as the obese rats were hyperinsulineamic and displayed normoglycaemia in comparison to lean controls. In addition, significant upregulation of markers of β-cell function was indicative of cells which were adapting or perhaps adapted to increased levels of circulating FFAs as opposed to dysfunction. Although morphological changes in the islets of the obese rats were not assessed in this study, Jones et al., reported that although the there was an increase in β-cell mass in ZO rats in comparison to lean controls, a minority of islets were degenerative (Jones, Nugent et al. 2010). It may be suggested that the upregulation of apoptotic markers as observed in the islets of the obese rats in this study occurs below an 'apoptosis threshold' and thus, results in the degeneration of a small minority of islets in the obese rats. In addition, the upregulation of pro-apoptotic markers in the islets of the obese rats was found to be accompanied by a concurrent upregulation of the anti-apoptotic BCL2 gene. The presence of opposing signals may result in degeneration in only a small

minority of susceptible cells within the islets. Furthermore, the nature of the ER stress induced in the islets of the obese rats is unclear. While chronic ER stress signalling is suggested to induce apoptosis, acute ER stress signals are suggested to mediate the propagation of cytoprotective signals (Marciniak, Ron 2006, Kaufman 2002). Whilst there was no evidence of dysfunction even in the presence of ER stress signals indicative of acute ER stress, the upregulation of apoptotic markers in the islets of the obese rats was indicative of chronic ER stress signalling. In addition, obesity develops over time and the sustained increase in plasma triglycerides as observed in the obese rats in this study would be expected to induce chronic ER stress (Sharma, Das et al. 2008). The ER stress response observed in this study may therefore have occurred early in the development of obesity or as a result of chronic ER stress. While the duration of the stress is undetermined, it is clear that an adaptive ER stress response occurred in the islets of the obese rats thus enabling the β-cells to increase their function in response to obesity and insulin resistance.

In contrast to the genetic rodent models of obesity, no significant increase in the expression of ER stress markers was observed in the islets of the DIO mice in comparison to lean controls. DIO mice characteristically develop dysregulation of insulin action and thus, insulin resistance (Surwit, Kuhn et al. 1988). Similar to the genetic rodent models of obesity, significant hyperinsulineamia was observed in the plasma of the DIO mice in comparison to lean controls, indicative of an increase in β-cell function in response to obesity and insulin resistance (**Figure 5.5b**). Despite the apparent increase in β-cell function, no significant upregulation was observed for the markers of ER stress assessed. A trend towards an increase was however observed in the expression of chaperone proteins such as calreticulin, HSPA5, HYOU1 and PDIA4 in the DIO mice islets in comparison to lean controls. This suggests that the use of more animals in each group may have resulted in statistical significance. In addition, the development of diet induced obesity follows a clinical course which varies widely from the monogenic model of genetic obesity. The gradual development of obesity and the much lower degree of insulin resistance in the DIO mice may therefore result in the activation of a much milder ER stress response in the islets of the DIO mice in comparison to genetic models of obesity.

5.3.3 ER stress signalling does not contribute to the development of β-cell dysfunction and apoptosis in diabetic female ZDF rats

The results discussed in this study so far indicate that an adaptive and perhaps, protective response is induced in the islets of genetically obese rats in comparison to their appropriate controls. While an increase in β-cell mass and function is a characteristic response to obesity, type 2 diabetes is characterised by progressive β-cell dysfunction which has been proposed to occur as a result of the detrimental effects of high levels of circulating FFAs and glucose in the blood plasma. Beta-cell dysfunction is evidenced by a significant decrease in circulating insulin levels accompanied by hyperglycaemia, indicating the inability of the β-cells to secrete adequate levels of insulin to maintain normoglycaemia. Several studies have reported a progressive decline in plasma insulin levels in the high fat fed female ZDFO rats in comparison to lean and chow fed ZDFO controls (Zhou, Madjidi et al. 2005, Teague, Gyte et al. 2010). In correlation with these studies, the high fat fed diabetic ZDFO (ZDFO-HFD) rats assessed in this study exhibited a significant and characteristic decline in plasma insulin levels coupled with hyperglycaemia, thus indicating β-cell dysfunction in the diabetic model in comparison to the obese yet euglycaemic chow fed ZDFO control rats. Transcriptional analysis of markers of β-cell function and differentiation further confirmed β-cell dysfunction in the islets of the diabetic rats with significant decline recorded for all markers assessed (**Table 5.4**). The significant downregulation of PDX1 and CCND1 in the islets of the diabetic rats is indicative of the loss of β-cell differentiation and growth in comparison to the obese non-diabetic rats. The significant downregulation of SLC2A2/GLUT2 and GCK, both under the regulatory control of PDX1, also went further to indicate the development of β-cell dysfunction in the islets of the diabetic rats. Other studies have also reported significant decline in the mRNA expression of GLUT2 and GCK in the islets and β-cells of human diabetic subjects and animal models of diabetes in comparison to lean and obese non-diabetic controls (Tokuyama, Sturis et al. 1995, Thorens, Wu et al. 1992, Gunton, Kulkarni et al. 2005).

While several other factors may occur, chronic induction of ER stress in response to elevated levels of FFAs has been implicated in development of β-cell dysfunction and

apoptosis in obesity associated type 2 diabetes (Laybutt, Preston et al. 2007). The upregulation of ER stress markers such as BiP/HSPA5, spliced XBP1 and CHOP has been reported in the islets of human diabetic subjects in comparison to non-diabetic controls (Laybutt, Preston et al. 2007, Huang, Lin et al. 2007, Marchetti, Bugliani et al. 2007), and in the islets of animal models of diabetes (Laybutt, Preston et al. 2007). While these studies showed evidence of ER stress, they failed to identify the contribution of ER stress to the development of β-cell dysfunction in the diabetic subjects. Having established the induction of an adaptive UPR in the islets of the chow fed normoglycaemic ZDFO rats in comparison to lean controls as previously discussed, it was surprising to observe no further increase in the expression of ER stress markers in the islets of the diabetic ZDFO rats in comparison to obese non-diabetic controls (**Table 5.4, Figure 5.7**). The lack of transcriptional regulation in the islets of the diabetic rats was further confirmed when protein expression was assessed by Western blotting with no potentiation in the protein expression of calreticulin, HYOU1 and GRP78/BiP in comparison to chow fed ZDFO controls (**Figure 5.7**). Despite this lack of UPR potentiation in the islets of the diabetic rats, the significant decline in plasma insulin levels was suggestive of β-cell dysfunction and perhaps, apoptosis. There was however, no potentiation in the expression of markers of apoptosis such as CHOP and caspase-12 in the islets of the diabetic rats in comparison to the obese non-diabetic rats (**Figure 5.7**).

The observations made in this study suggest that because of an antecedent compensation or adaptation in the islets in response to obesity, it was impossible to induce an additional compensatory response when further stress in the form of the high fat diet was introduced. A similar observation was made in a study in which culturing of 'compensated' islets isolated from prediabetic ZDF rats in FFA resulted in the inhibition of β-cell function and a reduction in pre-existing compensatory changes that had occurred *in vivo* (Hirose, Lee et al. 1996). The inability of pre-adapted cells to mount a further adaptive response was also demonstrated in a study by Rutkowski et al., in which MEF cells with low level ER stress were unable to mount a further stress response to the addition of another ER stress inducer (Rutkowski, Arnold et al. 2006). In consideration of these studies and the observations made in my study, one could

suggest that the development of β-cell dysfunction and apoptosis in obesity associated type 2 diabetes occurs as a result of the lack of a further adaptive response to ER stress as opposed to the chronic induction of ER stress signalling as is the general consensus.

While FFA induced ER stress may not play a significant role in the development of β-cell dysfunction in obesity associated type 2 diabetes, it is clear that placing the female ZDFO rats on a high fat diet was sufficient for the induction of β-cell dysfunction and subsequently, hyperglycaemia. This suggests that increased obesity and perhaps, the elevation of circulating FFAs, plays a role in the development of β-cell dysfunction. A recent study in ZDF rats reported the significant transcriptional downregulation of FA desaturases such as stearoyl-CoA desaturases (SCD) 1 and SCD2 and elongases such as fatty acid elongase 6 (ELOVL-6) in the islets of diabetic ZDF rats in comparison to prediabetic ZDF rats (Green, Olson 2011). Although a unique role for these enzymes in the pancreatic β-cell has not been identified, these enzymes function to desaturate and modify saturated fatty acids thus maintaining membrane FA composition and the generation of signalling molecules. The significant downregulation of these enzymes in the islets of the diabetic rats may result in the accumulation of saturated fatty acids such as palmitate, which may propagate cytotoxic signals through undetermined mechanisms. Knockdown of SCD in the INS-1 β-cell line in that study was found to result in a decrease in palmitate desaturation and an increase in palmitate induced ER stress and apoptosis (Green, Olson 2011). In addition, MIN6 cells expressing high levels of SCD1 were found to be more resistant to FFA-induced toxicity in comparison with low expressors of SCD1 (Busch, Gurisik et al. 2005). These studies suggest an alternative mechanism through which FFA may induce β-cell dysfunction and apoptosis in obesity associated type 2 diabetes. Other possible mechanisms include *de novo* ceramide synthesis and increased NO production (Shimabukuro, Zhou et al. 1998).

5.3.3. Conclusion

Data presented in this chapter provides evidence, in agreement with several other publications, of compensatory adaptation in the islets of obese rats in comparison to lean controls. It also suggests that while an adaptive ER stress response is activated in the islets of genetically obese rats; such a response is not induced in the islets of the high fat fed mouse model of insulin resistance. Additionally, despite the development of β-cell dysfunction in the diabetic ZDFO rat, there was no potentiation of ER stress signals, suggestive of the lack of ER stress involvement in the pathogenesis of type 2 diabetes. If chronic ER stress signalling is to be excluded as a major factor in the development of β-cell dysfunction and apoptosis in obesity associated type 2 diabetes, other possible pathways related to the metabolism and handling of FFAs must be investigated to determine the exact mechanisms involved in the pathogenesis of the disease.

CHAPTER 6

Chapter 6: Final Discussion

The development of type 2 diabetes (T2D) is characterised by progressive β-cell dysfunction in the context of insulin resistance in peripheral tissues. The failure of pancreatic β-cells to compensate for insulin resistance occurs due to defects in insulin secretion and/or the loss of β-cell mass as a result of apoptosis. Although not fully elucidated, the molecular mechanisms underlying β-cell dysfunction and apoptosis in T2D are beginning to emerge. Obesity, which is tightly linked to the development of insulin resistance, has been implicated in the pathogenesis of T2D. Elevated levels of plasma FFAs which can occur as a result of obesity, and exposure of β-cell lines to FFAs *in vitro* have been shown to cause dysfunction and apoptosis, thus suggesting a role for elevated FFAs in the development of β-cell dysfunction in obesity associated type 2 diabetes (Shimabukuro, Zhou et al. 1998, Cnop, Hannaert et al. 2001, Maestre, Jordan et al. 2003). More specifically, the induction of ER stress in response to elevated FFA levels has been proposed to play a significant role in the development of β-cell dysfunction and apoptosis. This thesis was thus aimed at investigating the role of ER stress in the development of β-cell dysfunction in obesity associated type 2 diabetes.

I demonstrate in Chapter 3, that an ER stress response is inducible in the MIN6 clonal β-cell line in response to thapsigargin. The UPR response to thapsigargin exposure was characterised by the phosphorylation and activation of PERK and IRE1, increased expression of ATF4 and increased XBP1 splicing. Although the proximal sensors of ER stress and their downstream targets were activated, the expression of chaperone proteins was generally unchanged although thapsigargin induced an increase in BiP expression. This was surprising as IRE1 signalling has been implicated in the upregulation of ER chaperone genes, together with the ATF6 signalling pathway, under ER stress conditions (Yoshida, Matsui et al. 2001). Although not assessed in this study, the lack of general upregulation in chaperone protein expression suggests that the ATF6 pathway was not activated, at least in response to thapsigargin exposure in our MIN6 cells. It also suggests that perhaps, IRE1 mediated chaperone protein upregulation is highly dependent on the activity of the ATF6 signalling pathway. As expected, chronic induction of ER stress as a result of thapsigargin mediated

irreversible ER Ca^{2+} depletion resulted in apoptosis as evidenced by CHOP and cleaved caspase-3 expression. The expression of CHOP in response to thapsigargin exposure was indicative of the activation of the PERK pathway in the MIN6 cells.

Both saturated and unsaturated FAs were also capable of inducing ER stress in MIN6 cells, albeit to different extents. In Chapter 3, I clearly showed that although both oleate and palmitate are capable of inducing ER stress, they differ greatly in their ability to promote apoptosis in the β-cell. In particular, the data presented shows that while both fatty acids were capable of inducing the expression of the pro-apoptotic marker CHOP; only palmitate readily promoted the loss of cell viability (**Chapter 3; Figure 3.6**). This confirms previously published reports that at least *in vitro*, long-chain saturated fatty acids are primarily responsible for the induction of β-cell apoptosis (Laybutt, Preston et al. 2007, Diakogiannaki, Welters et al. 2008). It remains unclear how long-chain saturated fatty acids exert their toxic effects on β-cells in comparison to unsaturated long-chain fatty acids. There however appears to be a critical difference in response which derives from the introduction of a single double bond into a long chain fatty acid molecule (Newsholme, Keane et al. 2007).

The data presented in Chapter 3 suggest that ER stress signalling does not contribute significantly to the induction of apoptosis in our MIN6 cells as CHOP expression in oleate treated cells did not result in apoptosis. It is apparent however, that CHOP plays a role in apoptotic signalling as deletion of the CHOP gene in Akita mice was reported to delay but not inhibit ER stress induced apoptosis (Oyadomari, Akio et al. 2002). In consideration of the report by Oyadomari et al., and the observations made in my study, it may be suggested that perhaps, a signalling partner is required for the pro-apoptotic effect of CHOP to be manifested in the β-cell. This would tie in with the observation that although CHOP was expressed in both oleate and palmitate treated cells, no loss of cell viability was observed in the oleate treated cells as there was perhaps, no apoptotic signalling partner with which CHOP could propagate it pro-apoptotic effects. Indeed, a recent study reported that the LIP isoform of C/EBPβ is required for the nuclear translocation and activation of CHOP in MEF cells under ER stress conditions (Chiribau, Gaccioli et al. 2010). Although the isoforms were not assessed, the protein expression of C/EBPβ in response to oleate and palmitate, and

the mRNA expression of C/EBPβ in response to palmitate only were detected in this study. While no significant changes were observed in C/EBPβ protein expression in response to both FFAs, palmitate induced significant upregulation of C/EBPβ transcription in MIN6 cells at all time points assessed (**Chapter 3, Figure 3.6di, dii**). In a study by Busch et al., palmitate but not oleate was reported to induce the mRNA expression of C/EBPβ in MIN6 cells (Busch, Cordery et al. 2002). In their study, the isoform of C/EBPβ was also not assessed. Although the contribution of the C/EBPβ isoforms to ER stress induced apoptosis is still debated, it would be interesting to determine which C/EBPβ isoforms are induced by palmitate exposure in MIN6 cells. Thus, it appears that palmitate toxicity may be mediated by a yet to be identified signalling mechanism which is required for the activation of CHOP. In conclusion, although both oleate and palmitate induced ER stress in this study, evidence suggests that palmitate induced apoptosis occurs through a mechanism which is not fully dependent of ER stress signalling in the MIN6 cells.

The upregulation of CHOP expression under ER stress conditions is dependent on ATF4, a transcription factor upregulated in response to eIF2α phosphorylation (Harding, Novoa et al. 2000b, Fawcett, Martindale et al. 1999). In Chapter 3 of this thesis as discussed above, I demonstrate that CHOP expression is upregulated in MIN6 cells exposed to thapsigargin, the unsaturated fatty acid oleate, and the saturated fatty acid palmitate, indicative of the activation of the PERK pathway in response to the ER stress inducers. Although the induction of apoptosis in the MIN6 cells appeared not to be solely dependent on ER stress induced CHOP expression as discussed above, it is clear that CHOP plays a role in apoptotic signalling in β-cells. As pro-apoptotic CHOP expression is induced downstream of the PERK pathway, the study described in Chapter 4 was designed to determine the role of PERK-dependent eIF2α phosphorylation in ER stress induced β-cell death. In Chapter 4, I provide evidence that eIF2α phosphorylation is not required for ER stress induced cell death as chronic dephosphorylation of eIF2α did not prevent thapsigargin or palmitate mediated apoptosis in the MIN6 cells. In fact, chronic dephosphorylation of basal eIF2α in control cells was found to be detrimental to cell survival in MIN6 cells (**Chapter 4, Figure 4.3**). This suggests that basal eIF2α phosphorylation and perhaps eIF2α phosphorylation in

response to ER stress, serve predominantly protective roles in ER stress signalling. Although chronic dephosphorylation of eIF2α was shown in this study to be detrimental to cell survival and to render cells more susceptible to ER stress induced apoptosis, the chronic phosphorylation of eIF2α has also been shown to exert toxic effects on the β-cell (Cnop, Ladriere et al. 2007). This indicates that the phosphorylation and dephosphorylation states of eIF2α in UPR signalling is subject to very delicate fine tuning and any artificial intervention in this process is likely to be poorly tolerated by the β-cell.

Under ER stress conditions, eIF2α phosphorylation results in the translational upregulation of ATF4, a transcription factor which in turn stimulates the expression of CHOP. It was therefore surprising to observe in MIN6 cells, that chronic dephosphorylation of eIF2α under ER stress conditions did not result in the inhibition of ATF4 and CHOP expression (**Chapter 4; Figure 4.2**). Indeed, this was in contrast to other similar studies in MEF cells (Scheuner, Song et al. 2001, Lu, Jousse et al. 2004). These data suggest that an alternative pathway independent of eIF2α phosphorylation, but perhaps dependent on the activation of PERK, may stimulate ATF4 and in turn, CHOP expression in MIN6 cells under ER stress conditions. ATF4 and CHOP have been reported to possess short half lives and as such, are rapidly degraded both at the protein and mRNA levels (Rutkowski, Arnold et al. 2006). The persistent upregulation of these transcription factors despite the chronic dephosphorylation of eIF2α was therefore suggestive of the activity of a positively perpetuating stress signal which was capable of sustaining their protein levels independent of eIF2α phosphorylation. As ATF4 expression has been shown to be dependent on the activation of PERK (Harding, Novoa et al. 2000b), I hypothesise that this novel pathway in the regulation of ATF4 and hence, CHOP expression is induced downstream of the PERK pathway. Thus, the expression of ATF4 in response to ER stress signals may be alternatively regulated by the activation of the Nrf2, the only other known PERK substrate (Cullinan, Zhang et al. 2003), independent of eIF2α phosphorylation. Indeed, Nrf2 has been shown to interact with ATF4, and may regulate its expression at least in retinal pigment endothelial cells (He, Gong et al. 2001, Miyamoto, Izumi et al. 2011). The potential role for Nrf2 in the regulation of ATF4 expression in β-cells remains to be investigated. Alternatively, the

possibility that ATF4 expression is co-regulated by a yet to be identified PERK substrate under ER stress conditions cannot be ruled out.

This thesis further aimed to characterise ER stress induction in the islets of animal models of obesity, and to determine the role played by this signalling pathway in the development of β-cell dysfunction in obesity associated type 2 diabetes. The results presented in Chapter 5 provide evidence for the induction of ER stress markers such as HSPA5/BiP, HYOU1 and calreticulin in the islets of rodent models of genetic obesity in comparison to their lean littermates (**Chapter 5; Figure 5.2**). ER stress induction in the islets of the obese rats was accompanied by compensatory adaptation in β-cell function and differentiation as indicated by transcriptional upregulation of markers such as INS2, IRS2, SLC2A2/GLUT2 and GCK. This suggests that the induction of ER stress serves a compensatory and perhaps, protective function in the islets of obese rats. The increases in the transcription of markers of β-cell function and differentiation and markers of cell cycle progression in the islets of the genetically obese rats were indicative of an attempt to increase β-cell mass and function in order to maintain normoglycaemia. This observation of compensatory adaptation correlates with several reports of increases in β-cell mass and function in the islets of obese animals and human subjects to compensate for insulin resistance in peripheral tissues as a result of obesity (Butler, Janson et al. 2003a, Matveyenko, Gurlo et al. 2009, Milburn, Hirose et al. 1995).

While ER stress and compensatory adaptation were apparent in the islets of genetically obese rats, it was surprising to observe no such changes occurred in the islets of DIO mice in comparison to lean controls. Although the DIO mice were capable of increasing insulin secretion in order to compensate for insulin resistance and to maintain glucose levels comparable to control mice, there was no evidence of significant ER stress induction in their islets (**Chapter 5; Figure 5.5**). This difference between the genetic rat models of obesity and the DIO mice may be a reflection of the different kinetics involved in the development of obesity between the two models. Diet induced obesity occurs at a more gradual pace than in genetic models, and results in much lower levels of insulin resistance (Ahrén, Pacini 2002). The resulting hyperinsulineamia, coupled with small increases in the expression of ER stress markers

in the islets of the DIO mice may thus reflect subtle changes which occur as obesity and insulin resistance progresses. This may thus, be a more accurate reflection of the transcriptional changes which may occur in the development of obesity and insulin resistance in humans. In addition, although considered a high fat diet, the 40 kcal% fat diet which the mice were placed on to induce obesity in this study, is reflective of the typical western diet which is rich in saturated (animal) fat. The subtle increases observed in the expression of ER stress markers in the islets of the DIO mice may thus reflect the changes which occur in the islets of human subjects in response to dietary habits in western society.

While obesity resulted in the induction of ER stress and adaptive changes in the islets of the normoglycaemic male ZO and female ZDFO rats, progression into β-cell dysfunction in the high fat fed female ZFDO rats was not accompanied by a concomitant rise the expression of ER stress markers. Indeed, significant downregulation was observed for markers of β-cell function and cell cycle progression such as INS2, SLC2A2/GLUT2, GCK, CCND1 and PDX1 in the islets of the obese diabetic rats in comparison to normoglycaemic obese control rats (**Chapter 5; Figure 5.4**). Downregulation of these genes, coupled with a significant decline in circulating insulin levels was indicative of β-cell dysfunction in the islets of the obese diabetic rats. The lack of ER stress potentiation in the islets of the diabetic rats despite the development of β-cell dysfunction, suggests that ER stress signalling does not contribute to the development of β-cell dysfunction in these animals. In fact, these data suggest that once a compensatory increase in the expression of ER stress markers occurs in response to obesity, the islets are unable to mount a further adaptive response when faced with further stress, in this case in the form of the high fat diet. The data in chapter 5 therefore suggest that β-cell dysfunction develops as a result of the inability of the β-cells to mount a further compensatory ER stress response to additional stress as opposed to chronic ER stress induction as is the general consensus.

Although this study was focused on ER stress induction in the islets of obese and/or diabetic animals, evidence of ER stress has also been reported in insulin sensitive peripheral tissues in animal models of obesity and human subjects (Umut, Qiong et al. 2004, Boden, Duan et al. 2008, Das, Chu et al. 2008). Indeed, ER stress has been

proposed to serve as the link between obesity and the development of insulin resistance in peripheral tissues (Umut, Qiong et al. 2004). ER stress through IRE1 activation, has been shown to directly trigger the activation of the JNK cascade (Urano, Wang et al. 2000). Activated IRE1 recruits the scaffolding protein TRAF2 to the ER membrane (Urano, Wang et al. 2000), resulting in the activation of a mitogen-activated protein (MAP) kinase cascade and consequently, JNK activation (Nishitoh, Saitoh et al. 1998, Nishitoh, Matsuzawa et al. 2002). Activated JNK promotes insulin resistance through the inhibitory phosphorylation of IRS-1 on serine residues (Umut, Qiong et al. 2004). Serine phosphorylation of IRS-1 inhibits its tyrosine phosphorylation by activated insulin receptors, thus impairing insulin signalling and resulting in insulin resistance. Evidence of JNK phosphorylation indicative of the development of insulin resistance in adipose tissue has been reported in several studies (Umut, Qiong et al. 2004, Boden, Duan et al. 2008). However, the phosphorylation of JNK was not observed in adipose tissue obtained from obese human subjects (Sharma, Das et al. 2008). Evidence for the induction of ER stress in peripheral tissues also includes the upregulation of BiP in the liver and adipose tissues of *ob/ob* mice and mice fed on a high fat diet (Umut, Qiong et al. 2004), and in the adipose tissue of diabetic *db/db* mice (Nakatani, Kaneto et al. 2005) in comparison to lean controls. Increases in the expression of BiP and other chaperone proteins have also been reported in the adipose and liver tissue of obese human subjects in comparison to lean controls (Boden, Duan et al. 2008, Sharma, Das et al. 2008). In agreement with these reports, increases in the mRNA expression of ER stress markers were observed in the adipose tissue of ZO rats in this study in comparison to their lean littermates (**Appendix 1.1a**), although the same was not observed in the adipose tissue of the female ZDFO rats in comparison to lean littermates (**Appendix 1.4a**). The expression of ER stress markers in the ZO adipose tissue is suggestive of the development of insulin resistance. Although phosphorylated JNK was not assessed, phosphorylated IRE1 was detected in the adipose tissue of the ZO rats in comparison to lean controls (**Appendix 1.1b**). Increased protein expression of ATF4 and GRP78/BiP was also detected in the ZO adipose in comparison to lean controls (**Appendix 1.1b**). In addition, although no significant changes in gene expression were observed in the liver of the ZO rats, significant IRE1 phosphorylation and GRP94 expression was detected by Western blotting in

comparison to lean controls (**Appendix 1.2**). The phosphorylation of IRE1 in the adipose and liver tissues of the ZO rats in comparison to lean controls is indicative of the induction of ER stress in the tissues and perhaps, the development of insulin resistance. Although unexpected, significant increases in the expression of ER stress markers was also detected in the muscle of the ZO rats in comparison to lean controls (**Appendix 1.3**). All together, the upregulation of ER stress markers and the phosphorylation of IRE1 in peripheral tissues in the ZO rats in comparison to their lean littermates confirms reports that ER stress and in particular, IRE1, serves as a plausible mechanistic link between obesity and peripheral insulin resistance.

In conclusion, the data in this thesis confirm that ER stress may be induced in β-cells *in vitro* in response to both saturated and unsaturated fatty acids, and *in vivo* in the islets of genetic models of rodent obesity. It also confirms the compensatory adaptation of islets, most probably β-cells, to insulin resistance in obese animals. While the significance of the PERK pathway in the induction of ER stress induced apoptosis is yet to be fully elucidated, data presented in this thesis suggests the existence of an alternative pathway in the regulation of ATF4 and thus, CHOP expression, downstream of PERK signalling. Work in this thesis also provides new evidence which suggests that the dysfunction of β-cells in obesity associated type 2 diabetes occurs by a mechanism which is independent of ER stress. Indeed, it appears that β-cell dysfunction develops as a result of an inability to mount an adaptive UPR in the islets of the diabetic animals.

APPENDIX 1

Appendix 1.1a. Adipose TLDA gene expression (male Zucker obese rats). Data obtained for all genes included in adipose TLDA and expressed as fold change compared to control Zucker lean rats. Data are normalised to housekeeping gene 18s.

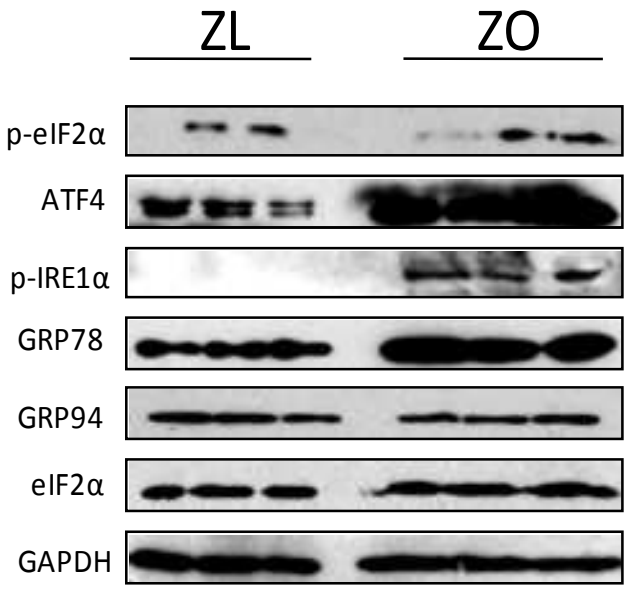
ER stress genes	Gene	ZO
Gene Name	Symbol	adipose
Hypoxia up-regulated 1	Hyou1	2.78
Wolframin	Wfs1	1.46
DnaJ (Hsp40) homolog, subfamily B, member 9	Dnajb9	2.45
CCAAT/Enhancer binding protein, beta	Cebpb	4.25
Nucleobindin	Nucb1	3.83
Protein disulphide isomerase family A, member 3	Pdia3	2.68
Paraoxonase 2	Pon2	2.61
Protein disulphide isomerase family A, member 4	Pdia4	4.13
Calnexin	Canx	3.45
UDP-glucose ceramide glucosyltransferase-like 1	Ugcgl1	3.89
Calreticulin	Calr	2.84
Growth arrest and DNA-damage-inducible, alpha	Gadd45a	14.80
Activating transcription factor 5	Atf5	2.69
Mitogen-activated protein kinase 8	Mapk8	4.19
PRKR-like endoplasmic reticulum kinase	Eif2ak3	1.59
Heat shock 70 kDa protein 5	Hspa5	3.99
Growth arrest and DNA-damage-inducible protein	Ddit3	4.47
Myeloid differentiation primary response gene 116	Myd116	2.84
DnaJ (Hsp40) homolog, subfamily C, member 3	Dnajc3	2.36
Activating transcription factor 4	Atf4	2.53
Homocysteine-inducible, ER stress-inducible	Herpud1	2.75
Tribbles 3	Trib3	5.55
X-box binding protein 1	Xbp1	-12.47
Quiescin Q6 sulfhydryl oxidase 1	Qscn6	1.71
FK506 binding protein 11	Fkbp11	1.07
Activating transcription factor 3	Atf3	10.35

β-cell function & differentiation genes	Gene	ZO
Gene Name	Symbol	adipose
Insulin 2	Ins2	-
Cyclin D1	Ccnd1	2.85
Glucose transporter type 2	Slc2a2/Glut2	-
Pancreatic and duodenal homeobox 1	Pdx1	-
Insulin Receptor Substrate 2	Irs2	2.46
Glucokinase/ Hexokinase 4	Gck	-

Lipid handling and metabolism genes	Gene	ZO
Gene Name	Symbol	adipose
Sterol Regulatory Element Binding Protein–1	Srebf1	5.4
HMG-CoA reductase	Hmgcr	2.85
Low density lipoprotein receptor	Ldlr	3.3
Fatty Acid Synthase	Fasn	23.32
Acetyl CoA Carboxylase	Acaca	6.15
Free fatty acid receptor 1	Ffar1	1.66

Apoptosis genes	Gene	ZO
Gene Name	Symbol	adipose
Bcl-2 homologous antagonist/killer	Bak1	4.33
B-cell lymphoma protein 2	Bcl2	15.29
Caspase 12	Casp 12	2.37
BCL2-associated X protein	Bax	3.40
BCL-X/BCL-2 binding protein	Bad	3.66

MIscellaeous genes	Gene	ZO
Gene Name	Symbol	adipose
Uncoupling protein 2	Ucp2	4.99



Appendix 1.1b. Detection of ER stress induction in obesity. Adipose tissues from ZO and ZL rats were solubilised in RIPA buffer and Western blotted using antisera against p-eIF2α, ATF4, p-IRE1α, XBP1s, GRP78, GRP94 and HYOU1. Total eIF2α and GAPDH were detected as protein loading controls. n=3 for each group.

Appendix 1.2a. Liver TLDA gene expression (male Zucker obese rats). Data obtained for all genes included in liver TLDA and expressed as fold change compared to control Zucker lean rats. Data are normalised to housekeeping gene 18s.

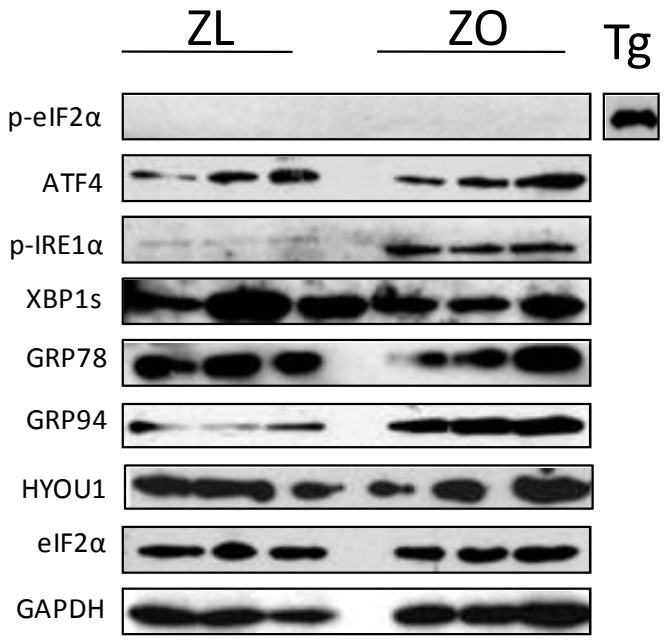
ER stress genes	Gene	ZO
Gene Name	Symbol	liver
Hypoxia up-regulated 1	Hyou1	-1.15
Wolframin	Wfs1	-1.68
DnaJ (Hsp40) homolog, subfamily B, member 9	Dnajb9	-1.48
CCAAT/Enhancer binding protein, beta	Cebpb	-1.31
Nucleobindin	Nucb1	-1.26
Protein disulphide isomerase family A, member 3	Pdia3	1.21
Paraoxonase 2	Pon2	1.05
Protein disulphide isomerase family A, member 4	Pdia4	1.09
Calnexin	Canx	1.06
UDP-glucose ceramide glucosyltransferase-like 1	Ugcgl1	-1.13
Calreticulin	Calr	1.10
Growth arrest and DNA-damage-inducible, alpha	Gadd45a	1.32
Activating transcription factor 5	Atf5	1.31
Mitogen-activated protein kinase 8	Mapk8	-1.14
PRKR-like endoplasmic reticulum kinase	Eif2ak3	1.45
Heat shock 70 kDa protein 5	Hspa5	1.00
Growth arrest and DNA-damage-inducible protein	Ddit3	1.26
Myeloid differentiation primary response gene 116	Myd116	-1.34
DnaJ (Hsp40) homolog, subfamily C, member 3	Dnajc3	-1.39
Activating transcription factor 4	Atf4	-1.06
Homocysteine-inducible, ER stress-inducible	Herpud1	-1.12
Tribbles 3	Trib3	1.17
X-box binding protein 1	Xbp1	-1.65
Quiescin Q6 sulfhydryl oxidase 1	Qscn6	-1.44
FK506 binding protein 11	Fkbp11	-1.56
Activating transcription factor 3	Atf3	-1.43

β-cell function & differentiation genes	Gene	ZO
Gene Name	Symbol	liver
Insulin 2	Ins2	-
Cyclin D1	Ccnd1	-2.11
Glucose transporter type 2	Slc2a2/Glut2	1.08
Pancreatic and duodenal homeobox 1	Pdx1	-
Insulin Receptor Substrate 2	Irs2	-2.38
Glucokinase/ Hexokinase 4	Gck	368

Lipid handling and metabolism genes	Gene	ZO
Gene Name	Symbol	liver
Sterol Regulatory Element Binding Protein–1	Srebf1	3.3
HMG-CoA reductase	Hmgcr	1.22
Low density lipoprotein receptor	Ldlr	1.27
Fatty Acid Synthase	Fasn	3.06
Acetyl CoA Carboxylase	Acaca	1.52
Free fatty acid receptor 1	Ffar1	1.03

Apoptosis genes	Gene	ZO
Gene Name	Symbol	liver
Bcl-2 homologous antagonist/killer	Bak1	-1.77
B-cell lymphoma protein 2	Bcl2	-1.63
Caspase 12	Casp 12	1.11
BCL2-associated X protein	Bax	-1.14
BCL-X/BCL-2 binding protein	Bad	-1.60

MIscellaeous genes	Gene	ZO
Gene Name	Symbol	liver
Uncoupling protein 2	Ucp2	-2.15



Appendix 1.2b. Detection of ER stress induction in obesity. Liver tissues from ZO and ZL rats were solubilised in RIPA buffer and Western blotted using antisera against p-eIF2α, ATF4, p-IRE1α, XBP1s, GRP78, GRP94 and HYOU1. Total eIF2α and GAPDH were detected as protein loading controls. A sample from MIN6 cells treated with 1μM thapsigargin for 2 h was used as a p-eIF2α antibody control. n=3 for each group.

Appendix 1.3. Muscle TLDA gene expression (male Zucker obese rats). Data obtained for all genes included in soleus muscle TLDA and expressed as fold change compared to control Zucker lean rats. Data are normalised to housekeeping gene 18s.

ER stress genes	Gene	ZO
Gene Name	Symbol	muscle
Hypoxia up-regulated 1	Hyou1	2.19
Wolframin	Wfs1	5.14
DnaJ (Hsp40) homolog, subfamily B, member 9	Dnajb9	4.10
CCAAT/Enhancer binding protein, beta	Cebpb	1.70
Nucleobindin	Nucb1	3.11
Protein disulphide isomerase family A, member 3	Pdia3	4.14
Paraoxonase 2	Pon2	3.08
Protein disulphide isomerase family A, member 4	Pdia4	3.32
Calnexin	Canx	4.38
UDP-glucose ceramide glucosyltransferase-like 1	Ugcgl1	2.32
Calreticulin	Calr	3.43
Growth arrest and DNA-damage-inducible, alpha	Gadd45a	2.01
Activating transcription factor 5	Atf5	2.02
Mitogen-activated protein kinase 8	Mapk8	4.19
PRKR-like endoplasmic reticulum kinase	Eif2ak3	-2.00
Heat shock 70 kDa protein 5	Hspa5	3.41
Growth arrest and DNA-damage-inducible protein	Ddit3	2.77
Myeloid differentiation primary response gene 116	Myd116	-1.15
DnaJ (Hsp40) homolog, subfamily C, member 3	Dnajc3	3.61
Activating transcription factor 4	Atf4	2.24
Homocysteine-inducible, ER stress-inducible	Herpud1	1.53
Tribbles 3	Trib3	-
X-box binding protein 1	Xbp1	1.24
Quiescin Q6 sulfhydryl oxidase 1	Qscn6	-1.50
FK506 binding protein 11	Fkbp11	-2.37
Activating transcription factor 3	Atf3	7.69

β-cell function & differentiation genes	Gene	ZO
Gene Name	Symbol	muscle
Insulin 2	Ins2	-
Cyclin D1	Ccnd1	2.32
Glucose transporter type 2	Slc2a2/Glut2	-
Pancreatic and duodenal homeobox 1	Pdx1	-
Insulin Receptor Substrate 2	Irs2	1.44
Glucokinase/ Hexokinase 4	Gck	-

Lipid handling and metabolism genes	Gene	ZO
Gene Name	Symbol	muscle
Sterol Regulatory Element Binding Protein–1	Srebf1	-1.34
HMG-CoA reductase	Hmgcr	-1.33
Low density lipoprotein receptor	Ldlr	3.24
Fatty Acid Synthase	Fasn	-1.19
Acetyl CoA Carboxylase	Acaca	-3.39
Free fatty acid receptor 1	Ffar1	3.11

Apoptosis genes	Gene	ZO
Gene Name	Symbol	muscle
Bcl-2 homologous antagonist/killer	Bak1	1.93
B-cell lymphoma protein 2	Bcl2	1.72
Caspase 12	Casp 12	1.66
BCL2-associated X protein	Bax	3.09
BCL-X/BCL-2 binding protein	Bad	-1.32

MIscellaeous genes	Gene	ZO
Gene Name	Symbol	muscle
Uncoupling protein 2	Ucp2	-1.2

Appendix 1.4a. Adipose TLDA gene expression in female ZDF rats. Data obtained for all genes included in adipose TLDA for chow fed ZDFO rats (table 1) and high fat fed ZDFO rats (table 2) expressed as fold change compared to control ZDF lean rats. Data are normalised to housekeeping gene 18s.

ER stress genes Gene Name	Gene Symbol	ZDFO adipose
Hypoxia up-regulated 1	Hyou1	1.12
Wolframin	Wfs1	1.46
DnaJ (Hsp40) homolog, subfamily B, member 9	Dnajb9	-1.04
CCAAT/Enhancer binding protein, beta	Cebpb	-1.52
Nucleobindin	Nucb1	1.31
Protein disulphide isomerase family A, member 3	Pdia3	1.50
Paraoxonase 2	Pon2	1.59
Protein disulphide isomerase family A, member 4	Pdia4	1.10
Calnexin	Canx	-2.13
UDP-glucose ceramide glucosyltransferase-like 1	Ugcgl1	1.16
Calreticulin	Calr	1.52
Growth arrest and DNA-damage-inducible, alpha	Gadd45a	3.59
Activating transcription factor 5	Atf5	1.45
Mitogen-activated protein kinase 8	Mapk8	1.26
PRKR-like endoplasmic reticulum kinase	Eif2ak3	1.60
Heat shock 70 kDa protein 5	Hspa5	1.66
Growth arrest and DNA-damage-inducible protein	Ddit3	1.09
Myeloid differentiation primary response gene 116	Myd116	-1.31
DnaJ (Hsp40) homolog, subfamily C, member 3	Dnajc3	1.25
Activating transcription factor 4	Atf4	1.18
Homocysteine-inducible, ER stress-inducible	Herpud1	1.34
Tribbles 3	Trib3	-2.42
X-box binding protein 1	Xbp1	-2.17
Quiescin Q6 sulfhydryl oxidase 1	Qscn6	1.19
FK506 binding protein 11	Fkbp11	1.69
Activating transcription factor 3	Atf3	1.68

β-cell function & differentiation genes Gene Name	Gene Symbol	ZDFO adipose
Insulin 2	Ins2	-
Cyclin D1	Ccnd1	1.33
Glucose transporter type 2	Slc2a2/Glut2	-
Pancreatic and duodenal homeobox 1	Pdx1	-
Insulin Receptor Substrate 2	Irs2	-1.47
Glucokinase/ Hexokinase 4	Gck	-

Lipid handling and metabolism genes Gene Name	Gene Symbol	ZDFO adipose
Sterol Regulatory Element Binding Protein–1	Srebf1	-1.18
HMG-CoA reductase	Hmgcr	1.55
Low density lipoprotein receptor	Ldlr	1.59
Fatty Acid Synthase	Fasn	1.89
Acetyl CoA Carboxylase	Acaca	-3.5
Free fatty acid receptor 1	Ffar1	-1.39

Apoptosis genes Gene Name	Gene Symbol	ZDFO adipose
Bcl-2 homologous antagonist/killer	Bak1	1.39
B-cell lymphoma protein 2	Bcl2	1.59
Caspase 12	Casp 12	1.92
BCL2-associated X protein	Bax	1.29
BCL-X/BCL-2 binding protein	Bad	1.18

MIscellaeous genes Gene Name	Gene Symbol	ZDFO adipose
Uncoupling protein 2	Ucp2	1.41

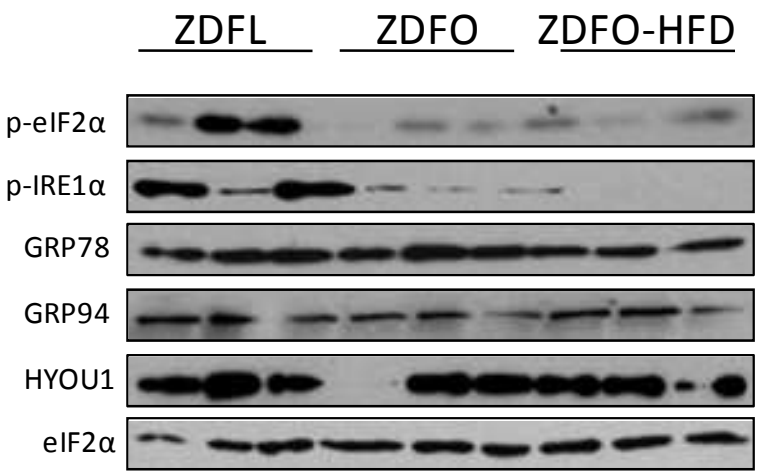
ER stress genes Gene Name	Gene Symbol	ZDFOD adipose
Hypoxia up-regulated 1	Hyou1	1.12
Wolframin	Wfs1	-1.28
DnaJ (Hsp40) homolog, subfamily B, member 9	Dnajb9	-1.34
CCAAT/Enhancer binding protein, beta	Cebpb	-2.25
Nucleobindin	Nucb1	-1.14
Protein disulphide isomerase family A, member 3	Pdia3	1.08
Paraoxonase 2	Pon2	1.16
Protein disulphide isomerase family A, member 4	Pdia4	-1.13
Calnexin	Canx	-2.74
UDP-glucose ceramide glucosyltransferase-like 1	Ugcgl1	-1.08
Calreticulin	Calr	1.12
Growth arrest and DNA-damage-inducible, alpha	Gadd45a	1.63
Activating transcription factor 5	Atf5	-1.57
Mitogen-activated protein kinase 8	Mapk8	-1.19
PRKR-like endoplasmic reticulum kinase	Eif2ak3	1.25
Heat shock 70 kDa protein 5	Hspa5	-1.26
Growth arrest and DNA-damage-inducible protein	Ddit3	-1.51
Myeloid differentiation primary response gene 116	Myd116	-1.92
DnaJ (Hsp40) homolog, subfamily C, member 3	Dnajc3	-1.08
Activating transcription factor 4	Atf4	-1.61
Homocysteine-inducible, ER stress-inducible	Herpud1	1.17
Tribbles 3	Trib3	-2.02
X-box binding protein 1	Xbp1	-3.29
Quiescin Q6 sulfhydryl oxidase 1	Qscn6	-1.54
FK506 binding protein 11	Fkbp11	-1.28
Activating transcription factor 3	Atf3	1.27

β-cell function & differentiation genes Gene Name	Gene Symbol	ZDFOD adipose
Insulin 2	Ins2	-
Cyclin D1	Ccnd1	-1.31
Glucose transporter type 2	Slc2a2/Glut2	-
Pancreatic and duodenal homeobox 1	Pdx1	-
Insulin Receptor Substrate 2	Irs2	-2.14
Glucokinase/ Hexokinase 4	Gck	-

Lipid handling and metabolism genes Gene Name	Gene Symbol	ZDFOD adipose
Sterol Regulatory Element Binding Protein–1	Srebf1	-2.16
HMG-CoA reductase	Hmgcr	1.42
Low density lipoprotein receptor	Ldlr	1.47
Fatty Acid Synthase	Fasn	-1.64
Acetyl CoA Carboxylase	Acaca	-7.54
Free fatty acid receptor 1	Ffar1	-1.1

Apoptosis genes Gene Name	Gene Symbol	ZDFOD adipose
Bcl-2 homologous antagonist/killer	Bak1	1.26
B-cell lymphoma protein 2	Bcl2	1.09
Caspase 12	Casp 12	-1.63
BCL2-associated X protein	Bax	-1.02
BCL-X/BCL-2 binding protein	Bad	-1.39

MIscellaeous genes Gene Name	Gene Symbol	ZDFOD adipose
Uncoupling protein 2	Ucp2	1.11



Appendix 1.4b. Detection of ER stress induction in obesity and diabetes. Adipose tissue from chow fed ZDFL, chow fed ZDFO and high fat fed ZDFO rats were solubilised in RIPA buffer and Western blotted using antisera against p-eIF2α, p-IRE1α, GRP78, GRP94 and HYOU1. Total eIF2α was detected as protein loading controls. n=3 for each group.

Appendix 1.5a. Liver TLDA gene expression in female ZDF rats. Data obtained for all genes included in liver TLDA for chow fed ZDFO rats (table 1) and high fat fed ZDFO rats (table 2) expressed as fold change compared to control ZDF lean rats. Data are normalised to housekeeping gene 18s.

ER stress genes Gene Name	Gene Symbol	ZDFO liver
Hypoxia up-regulated 1	Hyou1	1.59
Wolframin	Wfs1	1.31
DnaJ (Hsp40) homolog, subfamily B, member 9	Dnajb9	1.07
CCAAT/Enhancer binding protein, beta	Cebpb	1.55
Nucleobindin	Nucb1	1.26
Protein disulphide isomerase family A, member 3	Pdia3	1.03
Paraoxonase 2	Pon2	1.29
Protein disulphide isomerase family A, member 4	Pdia4	-1.31
Calnexin	Canx	-1.09
UDP-glucose ceramide glucosyltransferase-like 1	Ugcgl1	1.37
Calreticulin	Calr	-1.42
Growth arrest and DNA-damage-inducible, alpha	Gadd45a	1.55
Activating transcription factor 5	Atf5	1.95
Mitogen-activated protein kinase 8	Mapk8	1.63
PRKR-like endoplasmic reticulum kinase	Eif2ak3	-1.37
Heat shock 70 kDa protein 5	Hspa5	-1.14
Growth arrest and DNA-damage-inducible protein	Ddit3	1.71
Myeloid differentiation primary response gene 116	Myd116	1.29
DnaJ (Hsp40) homolog, subfamily C, member 3	Dnajc3	1.54
Activating transcription factor 4	Atf4	1.34
Homocysteine-inducible, ER stress-inducible	Herpud1	2.60
Tribbles 3	Trib3	-1.21
X-box binding protein 1	Xbp1	-1.13
Quiescin Q6 sulfhydryl oxidase 1	Qscn6	-1.99
FK506 binding protein 11	Fkbp11	2.07
Activating transcription factor 3	Atf3	-3.35

β-cell function & differentiation genes Gene Name	Gene Symbol	ZDFO liver
Insulin 2	Ins2	-
Cyclin D1	Ccnd1	-1.14
Glucose transporter type 2	Slc2a2/Glut2	2.05
Pancreatic and duodenal homeobox 1	Pdx1	-
Insulin Receptor Substrate 2	Irs2	-2.85
Glucokinase/ Hexokinase 4	Gck	12.01

Lipid handling and metabolism genes Gene Name	Gene Symbol	ZDFO liver
Sterol Regulatory Element Binding Protein–1	Srebf1	5.48
HMG-CoA reductase	Hmgcr	2.48
Low density lipoprotein receptor	Ldlr	1.39
Fatty Acid Synthase	Fasn	8.81
Acetyl CoA Carboxylase	Acaca	3.71
Free fatty acid receptor 1	Ffar1	6.92

Apoptosis genes Gene Name	Gene Symbol	ZDFO liver
Bcl-2 homologous antagonist/killer	Bak1	-1.11
B-cell lymphoma protein 2	Bcl2	1.05
Caspase 12	Casp 12	1.06
BCL2-associated X protein	Bax	1.05
BCL-X/BCL-2 binding protein	Bad	1.64

MIscellaeous genes Gene Name	Gene Symbol	ZDFO liver
Uncoupling protein 2	Ucp2	1.18

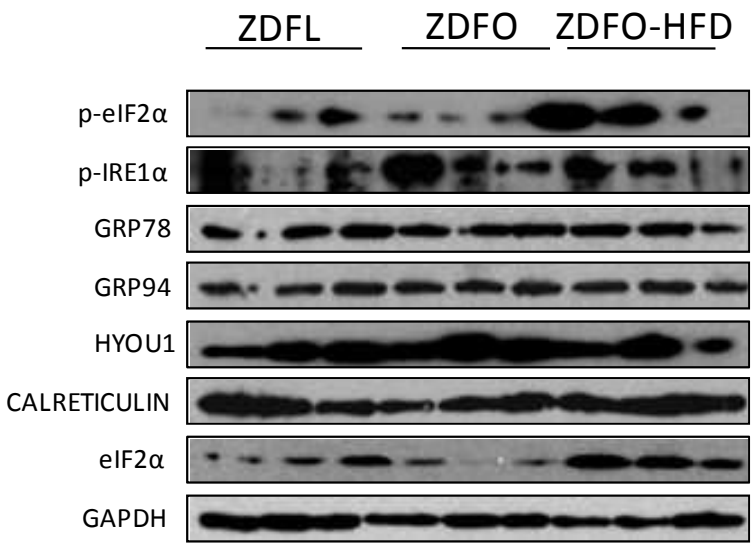
ER stress genes Gene Name	Gene Symbol	ZDFOD liver
Hypoxia up-regulated 1	Hyou1	-1.28
Wolframin	Wfs1	-1.75
DnaJ (Hsp40) homolog, subfamily B, member 9	Dnajb9	-1.10
CCAAT/Enhancer binding protein, beta	Cebpb	1.48
Nucleobindin	Nucb1	1.07
Protein disulphide isomerase family A, member 3	Pdia3	1.16
Paraoxonase 2	Pon2	-1.12
Protein disulphide isomerase family A, member 4	Pdia4	-1.17
Calnexin	Canx	-1.26
UDP-glucose ceramide glucosyltransferase-like 1	Ugcgl1	-1.02
Calreticulin	Calr	-1.06
Growth arrest and DNA-damage-inducible, alpha	Gadd45a	1.23
Activating transcription factor 5	Atf5	1.55
Mitogen-activated protein kinase 8	Mapk8	1.05
PRKR-like endoplasmic reticulum kinase	Eif2ak3	1.53
Heat shock 70 kDa protein 5	Hspa5	-1.03
Growth arrest and DNA-damage-inducible protein	Ddit3	1.86
Myeloid differentiation primary response gene 116	Myd116	-1.18
DnaJ (Hsp40) homolog, subfamily C, member 3	Dnajc3	1.26
Activating transcription factor 4	Atf4	1.09
Homocysteine-inducible, ER stress-inducible	Herpud1	3.25
Tribbles 3	Trib3	1.03
X-box binding protein 1	Xbp1	1.05
Quiescin Q6 sulfhydryl oxidase 1	Qscn6	-1.68
FK506 binding protein 11	Fkbp11	1.04
Activating transcription factor 3	Atf3	-5.01

β-cell function & differentiation genes Gene Name	Gene Symbol	ZDFOD liver
Insulin 2	Ins2	-
Cyclin D1	Ccnd1	-1.44
Glucose transporter type 2	Slc2a2/Glut2	1.27
Pancreatic and duodenal homeobox 1	Pdx1	-
Insulin Receptor Substrate 2	Irs2	-3.1
Glucokinase/ Hexokinase 4	Gck	111

Lipid handling and metabolism genes Gene Name	Gene Symbol	ZDFOD liver
Sterol Regulatory Element Binding Protein–1	Srebf1	6.9
HMG-CoA reductase	Hmgcr	-1.41
Low density lipoprotein receptor	Ldlr	-1.48
Fatty Acid Synthase	Fasn	1.48
Acetyl CoA Carboxylase	Acaca	1.45
Free fatty acid receptor 1	Ffar1	1.27

Apoptosis genes Gene Name	Gene Symbol	ZDFOD liver
Bcl-2 homologous antagonist/killer	Bak1	-1.02
B-cell lymphoma protein 2	Bcl2	-1.34
Caspase 12	Casp 12	-1.2
BCL2-associated X protein	Bax	1.01
BCL-X/BCL-2 binding protein	Bad	1.27

MIscellaeous genes Gene Name	Gene Symbol	ZDFOD liver
Uncoupling protein 2	Ucp2	1.02



Appendix 1.5b. Detection of ER stress induction in obesity and diabetes. Liver tissue from chow fed ZDFL, chow fed ZDFO and high fat fed ZDFO rats were solubilised in RIPA buffer and Western blotted using antisera against p-eIF2α, p-IRE1α, GRP78, GRP94, HYOU1 and calreticulin. Total eIF2α and GAPDH were detected as protein loading controls. n=3 for each group.

Appendix 1.6. Muscle TLDA gene expression in female ZDF rats. Data obtained for all genes included in soleus muscle TLDA for chow fed ZDFO rats (table 1) and high fat fed ZDFO rats (table 2) expressed as fold change compared to control ZDF lean rats. Data are normalised to housekeeping gene 18s.

ER stress genes Gene Name	Gene Symbol	ZDFO muscle
Hypoxia up-regulated 1	Hyou1	-2.26
Wolframin	Wfs1	-1.28
DnaJ (Hsp40) homolog, subfamily B, member 9	Dnajb9	1.09
CCAAT/Enhancer binding protein, beta	Cebpb	-2.08
Nucleobindin	Nucb1	-3.32
Protein disulphide isomerase family A, member 3	Pdia3	-3.46
Paraoxonase 2	Pon2	1.00
Protein disulphide isomerase family A, member 4	Pdia4	-2.91
Calnexin	Canx	-1.39
UDP-glucose ceramide glucosyltransferase-like 1	Ugcgl1	1.17
Calreticulin	Calr	-1.21
Growth arrest and DNA-damage-inducible, alpha	Gadd45a	-1.75
Activating transcription factor 5	Atf5	-1.92
Mitogen-activated protein kinase 8	Mapk8	-1.92
PRKR-like endoplasmic reticulum kinase	Eif2ak3	-1.08
Heat shock 70 kDa protein 5	Hspa5	1.20
Growth arrest and DNA-damage-inducible protein	Ddit3	-1.21
Myeloid differentiation primary response gene 116	Myd116	-3.50
DnaJ (Hsp40) homolog, subfamily C, member 3	Dnajc3	-1.69
Activating transcription factor 4	Atf4	-1.76
Homocysteine-inducible, ER stress-inducible	Herpud1	-1.27
Tribbles 3	Trib3	-
X-box binding protein 1	Xbp1	-1.45
Quiescin Q6 sulfhydryl oxidase 1	Qscn6	-1.61
FK506 binding protein 11	Fkbp11	-2.23
Activating transcription factor 3	Atf3	1.04

β-cell function & differentiation genes Gene Name	Gene Symbol	ZDFO muscle
Insulin 2	Ins2	-
Cyclin D1	Ccnd1	-1.5
Glucose transporter type 2	Slc2a2/Glut2	-
Pancreatic and duodenal homeobox 1	Pdx1	-
Insulin Receptor Substrate 2	Irs2	-1.8
Glucokinase/ Hexokinase 4	Gck	-

Lipid handling and metabolism genes Gene Name	Gene Symbol	ZDFO muscle
Sterol Regulatory Element Binding Protein–1	Srebf1	-1.41
HMG-CoA reductase	Hmgcr	-1.02
Low density lipoprotein receptor	Ldlr	-1.54
Fatty Acid Synthase	Fasn	-2.23
Acetyl CoA Carboxylase	Acaca	-1.18
Free fatty acid receptor 1	Ffar1	1.42

Apoptosis genes Gene Name	Gene Symbol	ZDFO muscle
Bcl-2 homologous antagonist/killer	Bak1	1.09
B-cell lymphoma protein 2	Bcl2	1.22
Caspase 12	Casp 12	-4.12
BCL2-associated X protein	Bax	-1.79
BCL-X/BCL-2 binding protein	Bad	-1.75

MIscellaeous genes Gene Name	Gene Symbol	ZDFO muscle
Uncoupling protein 2	Ucp2	-1.53

ER stress genes Gene Name	Gene Symbol	ZDFOD muscle
Hypoxia up-regulated 1	Hyou1	-3.17
Wolframin	Wfs1	-1.42
DnaJ (Hsp40) homolog, subfamily B, member 9	Dnajb9	1.30
CCAAT/Enhancer binding protein, beta	Cebpb	-1.75
Nucleobindin	Nucb1	-2.60
Protein disulphide isomerase family A, member 3	Pdia3	-5.00
Paraoxonase 2	Pon2	-1.25
Protein disulphide isomerase family A, member 4	Pdia4	-1.66
Calnexin	Canx	-1.53
UDP-glucose ceramide glucosyltransferase-like 1	Ugcgl1	-1.38
Calreticulin	Calr	1.03
Growth arrest and DNA-damage-inducible, alpha	Gadd45a	-1.11
Activating transcription factor 5	Atf5	-1.63
Mitogen-activated protein kinase 8	Mapk8	-1.63
PRKR-like endoplasmic reticulum kinase	Eif2ak3	-25.90
Heat shock 70 kDa protein 5	Hspa5	1.59
Growth arrest and DNA-damage-inducible protein	Ddit3	1.26
Myeloid differentiation primary response gene 116	Myd116	-3.16
DnaJ (Hsp40) homolog, subfamily C, member 3	Dnajc3	-1.44
Activating transcription factor 4	Atf4	-1.29
Homocysteine-inducible, ER stress-inducible	Herpud1	-1.17
Tribbles 3	Trib3	-
X-box binding protein 1	Xbp1	-1.91
Quiescin Q6 sulfhydryl oxidase 1	Qscn6	-1.33
FK506 binding protein 11	Fkbp11	-1.24
Activating transcription factor 3	Atf3	-1.27

β-cell function & differentiation genes Gene Name	Gene Symbol	ZDFOD muscle
Insulin 2	Ins2	-
Cyclin D1	Ccnd1	-1.86
Glucose transporter type 2	Slc2a2/Glut2	-
Pancreatic and duodenal homeobox 1	Pdx1	-
Insulin Receptor Substrate 2	Irs2	-1.97
Glucokinase/ Hexokinase 4	Gck	-

Lipid handling and metabolism genes Gene Name	Gene Symbol	ZDFOD muscle
Sterol Regulatory Element Binding Protein–1	Srebf1	-1.68
HMG-CoA reductase	Hmgcr	-1.45
Low density lipoprotein receptor	Ldlr	-1.81
Fatty Acid Synthase	Fasn	-2.31
Acetyl CoA Carboxylase	Acaca	-1.21
Free fatty acid receptor 1	Ffar1	-1.06

Apoptosis genes Gene Name	Gene Symbol	ZDFOD muscle
Bcl-2 homologous antagonist/killer	Bak1	1.36
B-cell lymphoma protein 2	Bcl2	2.04
Caspase 12	Casp 12	-2.74
BCL2-associated X protein	Bax	-1.38
BCL-X/BCL-2 binding protein	Bad	-1.27

MIscellaeous genes Gene Name	Gene Symbol	ZDFOD muscle
Uncoupling protein 2	Ucp2	-1.11

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