DEPARTMENT OF CLINICAL SCIENCE AND EDUCATION, SÖDERSJUKHUSET
Karolinska Institutet, Stockholm, Sweden

PROTEIN EXPRESSION ANALYSIS OF INSULIN PRODUCING CELLS EXPOSED TO ENDOPLASMIC RETICULUM STRESS

Victoria Rosengren

Karolinska Institutet

Stockholm 2015

Published by Karolinska Institutet. Printed by E-Print AB

ISBN 978-91-7549-802-7

Protein Expression Analysis of Insulin Producing Cells Exposed to Endoplasmic Reticulum Stress

THESIS FOR DOCTORAL DEGREE (Ph.D.)

by

Victoria Rosengren

Principal Supervisor:
Assoc Professor Henrik Ortsäter
Karolinska Institutet
Department of Clinical
Science and Education,
Södersjukhuset

Co-supervisor:
Henrik Johansson
Karolinska Institutet
Department of Oncology-Pathology
Science for Life Laboratory

Opponent:
Professor Nils Welsh
Uppsala University
Department of Medical Cell Biology

Examination Board:
Assoc Professor Anneli Björklund
Karolinska Institutet
Department of Molecular Medicine and Surgery

Professor Fredrik Palm
Linköping University
Department of Medical and Health Science
Division of Drug Research

Professor Jacob Odeberg
Royal Institute of Technology
School of Biotechnology
Science for Life Laboratory

With endless love to Mattias, Felix Vendela and Filip

”A very great vision is needed, and the man who has it must follow it as the eagle seeks the deepest blue of the sky” – Crazy Horse, Chief of the Sioux Indians

ABSTRACT

The prevalence of type 2 diabetes mellitus (T2DM) is increasing rapidly as a result of the obesity epidemic. T2DM develops in individuals who fails to compensate for increased demand of insulin. Obese individuals display alterations in the circulating lipid profile and there is evidence that the elevated levels of fatty acids are detrimental to pancreatic β-cells which may contribute to the progression of β-cell failure via both impaired function and a reduction of the β-cell mass seen in T2DM patients, but the mechanism is not completely known. There is evidence that saturated fatty acids can induce apoptosis, as well as endoplasmic reticulum (ER) stress. As a secretory cell, the pancreatic β-cell is equipped with a highly developed ER to assist in the folding process of newly synthesized proteins, but this cell organelle is sensitive to alteration in homeostasis, and an imbalance between protein load and folding capacity can result in ER stress. This thesis aimed to investigate the protein expression in insulin producing cells exposed to ER stress by the use of proteomics and Western blot.

In **paper I**, cells were exposed to the ER stress inducer thapsigargin. By the use of proteomic methodology we found decreased levels of the ER localized proteins GRP78/BiP, PDIA3 and PDIA6 following thapsigargin treatment. The decreased protein levels of GRP78/BiP was not a concequence of reduced mRNA expression. Instead the decrease involved a combination of reduced protein synthesis and enhanced degradation by both proteasome and autophagy. The data in paper I provides an explanation to why INS-1E cells are vulnerable to conditions of ER stress. In **paper II**, cells were exposed to thapsigargin and the saturated fatty acid palmitate. By proteomic investigation we identified 7,786 proteins, of which 6,117 were overlapping in two data sets. 1,000 proteins were regulated by thapsigargin and 227 by palmitate. With bioinformatic analysis we found a set of transcription factors predicted to be regulated in the same manner by the thapsigargin and palmitate. This paper shows that the treatments not only share the induction of ER stress but also share an effect on the lipid handling in the cell by affecting several metabolic master transcription factors involved in lipid and cholesterol biosynthesis. In **paper III** we investigated pathways involved in the cytotoxic action of glucocorticoids (GCs). Excess of GCs is associated with glucose intolerance and diabetes. The data show that GC activates p38 MAPK and JNK in β-cells, which work in opposite to regulate the cytotoxic effects induced by GCs. The data also suggests that protein phosphatase 5 play a protective role, since reduced PP5 levels in MIN6 cells and islets makes the cells sensitized to the toxic effects induced by GCs. Furthermore, our result indicate that GC exposure

compromises the cellular response to ER stress in insulin producing cells which may lead to an impaired capacity to restore the homeostasis in the ER A deeper understanding of the mechanisms and the mediators of the ER and apoptotic pathways will hopefully provide tools to preserve the β-cell mass and delay the progression to T2DM.

LIST OF PUBLICATIONS

Victoria Rosengren, Henrik Johansson, Janne Lehtiö, Liselotte Fransson, Åke Sjöholm, Henrik Ortsäter. Thapsigargin down-regulates protein levels of GRP78/BiP in INS-1E cells.
Journal of Cellular Biochemistry, 2012, 113(5), 1635-1644.

Victoria Rosengren, Henrik Johansson, Janne Lehtiö, Henrik Ortsäter. Metabolic Master Transcription Factors affected by treatment with Palmitate and Thapsigargin - A Proteomic Investigation of the Insulin-Producing Cell Line INS-1E.
Submitted.

Liselotte Fransson, **Victoria Rosengren**, Titu Kumar Saha, Nina Grankvist, Tohidul Islam, Richard E. Honkanen, Åke Sjöholm, Henrik Ortsäter. Mitogen-activated protein kinases and protein phosphatase 5 mediate glucocorticoid-induced cytotoxicity in pancreatic islets and β-cells.
Molecular and Cellular Endocrinology, 2014, 383, 126-136.

Publication not included in the thesis

Liselotte Fransson, Stephanie Franzén, **Victoria Rosengren**, Petra Wolbert, Åke Sjöholm, Henrik Ortsäter. β-cell adaptation in a mouse model of glucocorticoid-induced metabolic syndrome.
Journal of Endocrinology, 2013, 219(3), 231-241.

CONTENTS

LIST OF ABBREVIATIONS

2DGE	Two-dimension gel electrophoresis
3-MA	3-methyladenine
ATF6	Activating transcription factor 6
ATF4	Activating transcription factor 4
BSA	Bovine serum albumin
CHOP	CCAAT/enhancer binding protein homologous protein
CID	Collision induced dissociation
CPA	Cyclopiazonic acid
DMEM	Dulbecco's Modified Eagle Medium
ECL	Enhanced chemiluminescence
eIF2α	Eukaryotic translation initiation factor 2, α subunit
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ERAD	ER associated degradation
ESI	Electrospray ionization
FAs	Fatty acids
FBS	Fetal bovine serum
FOXO1	Forkhead box protein O1
FOXM1	Forkhead box protein M1
GADD34	Growth arrest and DNA damage-inducible protein 34
GC	Glucocorticoids
GDM	Gestational diabetes mellitus
GLUT	Glucose transporter
GO	Gene Ontology
GR	Glucocorticoid Receptor
GSIS	Glucose-stimulated insulin secretion
HCD	Higher-energy collisional dissociation
IEF	Isoelectric focusing
pI	Isoelectric point
IPG	Immobilized pH gradient
IR	Insulin receptor
IRS	Insulin receptor substrate
IRE1	Inositol-requiring enzyme 1
iTRAQ	Isobaric tag for relative and absolute quantification

JNK	c-Jun N-terminal kinase
LC	Liquid chromathography
LTQ	Linear quadropole ion trap
MALDI	Matrix assisted laser desorption ionization
MAPK	Mitogen activated protein kinase
mRNA	Messenger RNA
MS	Mass spectrometry
PBS	Phosphate Buffered saline
PCR	Polymerase chain reaction
PDI	Protein disulfide-isomerase
p-eIF2α	Phosphorylated eukaryotic translation initiation factor 2, α-subunit
PERK	PKR-like ER localized eIF2α kinase
PI3K	phosphatidylinositol-3-kinase
PP5	Protein phosphatise 5
PPARA	Peroxisome proliferator-activated receptor alpha
qPCR	Quantitative PCR
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SERCA	Sarco/endoplasmic reticulum Ca^{2+}-ATPase
siRNA	Small interfering RNA
SREBP-1	Sterol regulatory element-binding protein 1
SREBP-2	Sterol regulatory element-binding protein 2
SVA	Statens veterinärmedicinska anstalt
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TCA cycle	Tricarboxylic acid cycle
TOF	Time of flight
UPR	Unfolded protein response
WHO	World Health Organization
XBP1	X-box binding protein 1
XBP1s	Spliced XBP1

1 INTRODUCTION

1.1 THE HISTORY OF DIABETES MELLITUS – FROM ANCIENT TIMES TO THE PRESENT

Diabetes Mellitus is far from a new disease. Even the ancient Egyptians were suffering from it. The earliest known reference to what could be a description of diabetes mellitus was found in an Egyptian medical document called the Ebers Papyrus, written 1500 B.C. The hieroglyphs in this document describe a condition of "too great emptying of the urine" which could be the symptom of the excessive urination (polyuria) seen in patients suffering from diabetes mellitus. Around the same time in India, physicians observed that urine from patients with polyuria attracted ants and flies, and named the condition "honey urine" [1]. Today we know that these are symptoms of a pronounced lack of insulin leading to high levels of glucose in the blood. The name of the disease comes from the symptoms of the patient; Diabetes, which in Greek means "to pass through", referring to the excessive amount of urine, and Mellitus from "honey-sweet", referring to the excessive amount of sugar in the blood and urine.

Before the discovery of insulin, diabetes mellitus was a deadly disease and it was not until the 19th century the first step to this discovery was done. In 1869 Paul Langerhans described the histological structure of the pancreatic islets, but the function of these islets remained unknown for some years. When Oscar Minowski, in 1889, observed that removal of pancreas from dogs made them diabetic he established the connection between this organ and the control of blood glucose. The main breakthrough came when Frederick Banting and Charles Best in 1921 were able to extract insulin from pancreatic islets, and in 1922 the first diabetic patient, 14-year old Leonard Thompson, was injected with the extract. In 1923 the Nobel Prize was awarded for the discovery of insulin. Today insulin is the only medical treatment for people with type 1 diabetes mellitus (T1DM), the second most common form of diabetes, while people with type 2 diabetes mellitus (T2DM), the most common form of diabetes, may need insulin in later stages of the disease.

Even though diabetes mellitus has been known for more than 3000 years the exact mechanism for the cause of the disease is still not known. In earlier years, T1DM was the mainly form of diabetes, but with the increased prevalence of obesity the number of individuals diagnosed with T2DM have bypassed the number of T1DM. The prevalence of diabetes has been rapidly increased in the last decades and the number of people with the disease has

more than doubled during the past 20 years. Today 382 million people are estimated to suffer from diabetes, a number predicted to increase to almost 600 million by the year 2035 [2, 3], but still, 175 million cases are predicted to be undiagnosed [2]. Chronic hyperglycemia in undiagnosed people, or poorly managed diabetes in patients, is associated with severe and long-term complications affecting several organs including eyes, kidneys, nerves, heart, and blood vessels [2]. In addition to the suffering for affected individuals, the economic burden of diabetes is enormous to health care systems.

In later years, development of new techniques has brought knowledge of the development of diabetes forward by investigation of the genome and proteome. Genome-wide association studies have identified many susceptibility genes linked to diabetes mellitus, many of them expressed in pancreatic β-cells showing the importance for these insulin producing cells in the development of T2DM. With different proteomic methods it is possible to simultaneously analyze virtually all proteins expressed in a cell, and with these unbiased approaches it is possible to reveal novel findings that otherwise had not been identified, if a single pathway or a set of proteins decided beforehand, had been investigated. The aim of the present work was to add a few pieces to the complex puzzle underlying the development of T2DM by the use of proteomics in cells exposed to endoplasmic reticulum stress.

2 BACKGROUND

2.1 DIABETES MELLITUS

Diabetes is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action or a combination of both [2]. The disease is classified into four different categories based on etiology and clinical features; **I)** Type 1 diabetes mellitus (T1DM), **II)** Type 2 diabetes mellitus (T2DM), **III)** Other specific types of diabetes, and **IV)** Gestational diabetes mellitus (GDM) [4]. T1DM, also called insulin-dependent diabetes, results from an autoimmune destruction of the insulin producing β-cells in pancreas leading to an absolute deficiency of insulin. The patients suffering from T1DM are therefore dependent on exogenous administration of insulin for their survival. The onset of disease usually occurs at young age. T2DM, also called non-insulin dependent diabetes, is the largest form of diabetes, accounting for ~90-95% of all cases of diabetes. Since the present thesis is focusing on T2DM, this category is further described in the next section. Other specific types of diabetes include genetic defects of the β-cell function or insulin actions, diseases of the exocrine pancreas, endocrinopathies, or induced by drugs or chemicals. GDM is diagnosed during pregnancy after development of resistance to insulin and subsequent hyperglycemia [4].

2.1.1 Type 2 diabetes mellitus

As mentioned earlier, T2DM is the most common type of diabetes, nowadays accounting for ~90-95% of all cases of diabetes. The disease is usually associated with a relative lack of insulin (rather than an absolute deficiency as in the case of T1DM), often in combination with insulin resistance. Before diagnosis of T2DM, individuals can remain undiagnosed for many years because of the gradually developing hyperglycemia, which increases the risk for complications due to the long-term exposure to elevated glucose levels in the circulation. At the pre stage of the disease the circulating insulin levels can be high to compensate for a reduced insulin response. As the disease progress, the function of the β-cells become impaired and when the level of insulin not is enough to maintain normoglycemia, T2DM is a fact [4]. According to recommendations from World Health Organization (WHO) diabetes is diagnosed if the fasting plasma glucose is ≥7 mmol/L or the plasma glucose concentration is ≥11.1 mmol/L two hours after an oral glucose load [5]. Clinical management of T2DM includes a combination of lifestyle interventions, with changes in diet

and increased physical activity [6], and pharmacological treatment which aims to increase the insulin secretion or increase the insulin sensitivity in peripheral tissue [7, 8]. In later stages of the disease, the patients may need insulin to control their blood glucose levels.

The aetiology of T2DM is poorly understood but it results from a complex interplay between genetic predisposition and environmental factors. Genomic studies have so far identified approximately 70 genomic regions associated with T2DM [9, 10], but all of them together only explain a small part of the genetic background of the disease [11]. Most of these identified genes have been shown to be involved in pancreatic β-cell mass and/or function, associated to insulin secretion defects [12], showing the importance for these insulin producing cells in the development of T2DM. Environmental factors contributing to the development of the disease includes high caloric intake and a sedentary lifestyle leading to obesity, and as in the case of T2DM, the prevalence of obesity is also increasing across the world [13]. Obesity is strongly associated with increased risk of developing insulin resistance which leads to loss of insulin response in peripheral tissue [14, 15]. T2DM is a disease most common among adults but a worrying aspect is the rapid increase of T2DM in children and adolescents. As in adults, obesity and insulin resistance are risk factors for paediatric T2DM [16, 17]. Alterations in the circulating lipid profile have been observed in obese individuals [18, 19] and there is mounting evidence that the elevated levels of circulating free fatty acids can be detrimental to pancreatic β-cells which during long term effect may contribute to the progression of β-cell failure and T2DM [20-22].

2.1.2 The pancreatic islet and the insulin-producing beta-cell

The pancreas is an organ consisting of both endocrine and exocrine tissue where the endocrine pancreas makes up only a small part of the whole pancreas (1-2% of total pancreas mass). These endocrine cells are organized in micro-organs called the islets of Langerhans. About one million islets are scattered throughout the pancreatic gland. The human islets consist of five different cell types with the insulin-producing β-cell representing the major part of the total cell mass in the islet [23, 24]. The remaining endocrine cell types in the islet are the glucagon-producing α-cell, the somatostatin-producing δ-cell, the pancreatic polypeptide-producing PP-cell, and the ghrelin-producing ε-cell [25]. Together these cells cooperate to maintain the glucose homeostasis.

Approximately 50-80% of the cells in the islets are β-cells [25]. The β-cells develop from endocrine precursor cells that differentiate from epithelial progenitor cells in pancreatic ducts during prenatal life [26]. In humans the majority of β-cell expansion occurs in early childhood [27] where the highest rate of proliferation has been observed before 2 years of age [28, 29]. During adulthood the β-cell mass is highly adaptive to changes in metabolic homeostasis. When increased demand for insulin exists, such in the case of obesity [30], hyperglycemia or pregnancy [19], expansion of the β-cell mass has been shown to occur, to be able to secrete more insulin.

2.1.3 Insulin synthesis and release

Insulin is the key hormone required for maintenance of glucose homeostasis and as mentioned above, it is produced in and secreted from the β-cells in pancreas. The secretion of insulin is stimulated by intracellular signals obtained from metabolism of nutrients where glucose is the primary stimulus [31].

The human gene for insulin is located on chromosome 11. The synthesis of insulin starts with transcription of the insulin gene and subsequent translation of insulin mRNA into preproinsulin in the endoplasmic reticulum (ER) [1]. Insulin-producing β-cells (as well as other secretory cells) have a highly developed ER to meet the demand for the large amount of proteins to be synthesized by the cell [32]. In the ER, preproinsulin is cleaved to proinsulin by removal of the signal peptide. Proinsulin is packed into vesicles and transported to the *trans* Golgi network where it is converted to mature insulin by the action of prohormone-converting enzymes (PC1/3 and PC2). The mature insulin, in a crystalline form of six insulin molecules and two zinc atoms, and the C-peptide are stored in the granules until the β-cell get signals to release the content into the bloodstream [1].

Glucose is the main physiological regulator of insulin secretion in pancreatic β-cells, but the cells also respond to other nutrients such as amino acids and fatty acids. The glucose-stimulated insulin secretion (GSIS) generates a triggering and an amplifying pathway in the cell (also called the K_{ATP}-dependent and K_{ATP}-independent pathways) [31, 33]. When the level of glucose in the blood is high, the triggering pathway starts with entering of glucose through specific glucose transporters (GLUT-1 in human and GLUT-2 in rodents) in the β-cell [34, 35]. Glucose is then rapidly phosphorylated by glucokinase and further metabolized to pyruvate in series of enzyme-catalysed reactions called the glycolysis. Pyruvate enters the mitochondria and is further metabolized in the tricarboxylic acid cycle (TCA cycle),

generating NADH and $FADH_2$ which are used in the respiratory chain, for adenosine triphosphate (ATP) production. The increased ATP/ADP (adenosine diphosphate) ratio leads to closure of the K_{ATP} channels which result in membrane depolarisation and activation of voltage-dependent Ca^{2+} channels. Influx of Ca^{2+} into the cell increases the levels of cytoplasmic free Ca^{2+} which triggers fusion of a readily releasable pool (RRP) of insulin-containing vesicles with the plasma membrane, resulting in a rapid release of insulin [36, 37]. This triggering mechanism is mainly responsible for a first phase of insulin secretory response, starting within minutes after glucose stimulation and lasts for 5-10 minutes [38] (Fig 1). When the RRP of insulin-containing vesicles is depleted, it is refilled from a reserve pool (RP).

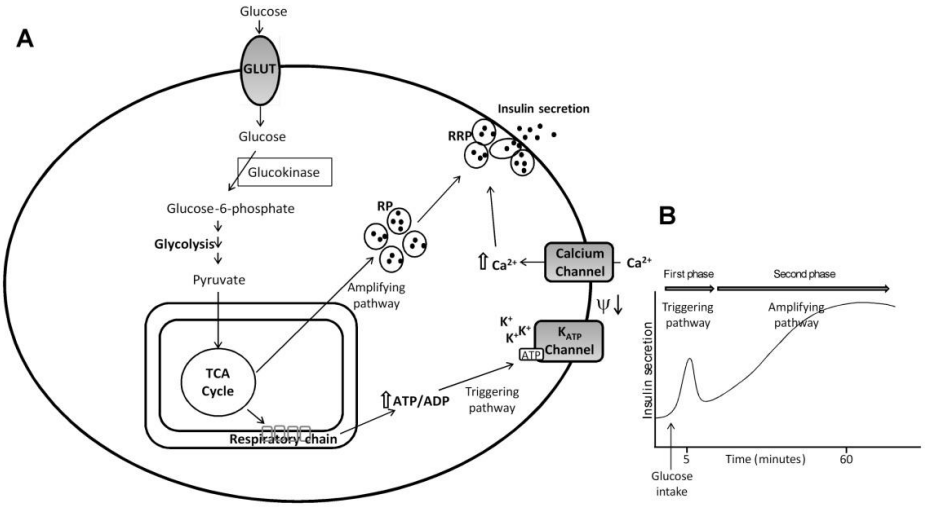

Figure 1. Glucose-stimulated insulin secretion in the β-cell. (A) In the triggering pathway, glucose is transported into the cell through GLUT-1 (humans) or GLUT-2 (rodents) and is metabolized to pyruvate which enters the mitochondria. In the TCA cycle ATP is yielded and the increased ATP/ADP ratio closes K_{ATP} channels. The subsequent depolarization of the membrane result in activation of Ca^{2+} channels, influx of Ca^{2+} and insulin release. The amplifying pathway augments the insulin secretion independent of the K_{ATP} channel. RRP (readily releasable pool), RP (reserve pool). **(B)** Insulin is secreted from the β-cell in a biphasic manner after glucose intake. The initial phase is rapid and dependent on generation of ATP and the subsequent influx of Ca^{2+} in the triggering pathway. The second, prolonged phase is dependent on mitochondrial metabolism in the amplifying pathway.

The amplifying pathway of GSIS augments the secretory response of insulin independent of the K_{ATP} channels. This pathway is mainly responsible for a second, more sustained, phase of insulin secretion which is gradually

increased over a period of 60 minutes [37]. The mechanisms involved in the amplifying pathway are not fully understood but several factors have been suggested where anaplerotic metabolism of pyruvate and efflux of TCA cycle intermediates are suggested as key events in this process [33, 39-41] (Fig 1).

In healthy individuals, there is a balance between insulin secretion from pancreatic β-cells and insulin action in peripheral tissue to keep the blood glucose concentration within normal range (~5 mM). Several steps in the GSIS have been revealed to be impaired in type 2 diabetic patients. Loss of first phase of insulin secretion is an early pathogenic event in T2DM [42] and reduced insulin secretion from isolated islets from type 2 diabetic patients have been observed [43] as well as reduced expression of glucokinase in islets from T2DM donors [44]. As mentioned before, of the susceptibility genes associated with T2DM, most of these genes have been shown to be involved in pancreatic β-cell mass and/or function, associated to insulin secretion defects [12, 43].

2.1.4 Insulin signaling and regulation of blood glucose levels

The main purpose of the insulin secreted by the pancreatic β-cells is to promote uptake of glucose in peripheral tissue and to suppress glucose production in the liver. Insulin initiates its biological actions by binding to the transmembrane insulin receptor (IR) on the surface of the target cell. The IR consists of two α-subunits and two β-subunits which are linked by disulphide bonds. Binding of insulin to the α-subunit results in autophosphorylation of the β-subunit and activation of insulin receptor substrate (IRS) 1 and 2 which can activate the phosphatidylinositol-3-kinase (PI3K) pathway, leading to translocation of glucose transporter 4 (GLUT-4) to the surface of the target cell. GLUT-4 is responsible for glucose transport from the blood into skeletal muscle and adipose tissue [1, 45]. Insulin and the glucose uptake stimulate glycogen synthesis in skeletal muscle, and promote lipogenesis and inhibit lipolysis in adipose tissue [15].

2.2 PANCREATIC BETA-CELL DYSFUNCTION

Pancreatic β-cell dysfunction is central in T2DM pathogenesis [46] and is often associated with peripheral insulin resistance. Reduced sensitivity to insulin leads to an increased demand on the β-cell to secrete insulin in order to maintain glucose homeostasis. T2DM develops when the β-cell no longer can meet the secretory demand to maintain normoglycemia. Several mechanisms have been suggested to be involved in the progression of the

reduced β-cell function and β-cell mass and the mechanisms relevant to this work are describe below.

2.2.1 Lipotoxicity in pancreatic beta-cells

Lipids are normally stored in adipose tissue. When the supply of fatty acids (FA) overwhelms the storage capacity of adipose tissue, lipids accumulates in tissues other than adipose tissue, such as pancreas [47], where they exert harmful effects, a condition termed lipotoxicity [48]. Circulating free FAs are elevated in obese individuals [18, 19] and there is evidence that the elevated systemic levels of FAs are detrimental to pancreatic β-cells which may contribute to the progression of β-cell failure via both impaired function and reduction in β-cell mass seen in T2DM patients [21, 22, 49, 50].

Different fatty acids have different effects on the β-cells, where long-chain saturated FAs have been shown to be more toxic than both shorter saturated FAs and unsaturated FAs [50-52]. Palmitate (C16:0) is the most common saturated FA in plasma [18] and is therefore often used for investigation of the cytotoxic effects induced by FAs. The mechanisms causing the lipotoxic β-cell dysfunction and death are not completely understood, but several pathways have been shown to be affected, including ER stress [53] and autophagy [54]. In addition to the lipidemic environment in obesity, the progression to T2DM is also associated with high levels of glucose, leading to a glucolipotoxic environment for the β-cell [55, 56]. The glucolipotoxicity is associated with oxidative stress, induced by reactive oxygen species (ROS), generated during intracellular metabolism of glucose in the mitochondria which may cause damage to the mitochondrial DNA [57]. In addition, when glucose level is high, metabolism of glucose leads to flux of citrate from mitochondria and generation of malonyl-CoA in the cytosol. The increased level of malonyl-CoA inhibits the mitochondrial FA transporter carnitine palmitoyltransferase-1 (CPT-1) activity and stops FAs to enter the mitochondria for fatty acid β-oxidation. Instead, long-chain acyl-CoA accumulates in the cytosol where they directly or via generation of lipid derived signals negatively affects the β-cell function [50].

2.2.2 Apoptosis of pancreatic beta-cells

Programmed cell death, or apoptosis, is a regulated process leading to death of the cell. Apoptosis is important for maintenance of cellular homeostasis during development but is also a defense mechanism to remove damage cells [58]. Apoptotic cell death is triggered by an extrinsic, receptor-mediated signaling pathway or an intrinsic, mitochondrial-

mediated signaling pathway. The intrinsic pathway of apoptosis has been shown to be initiated by several insults, including DNA-damage, dysfunction of mitochondria (due to ROS or Ca^{2+} overload) and ER stress [58]. The protease family of caspases is the executors of cell death, but caspase-independent mechanisms also exists [59].

The β-cell mass has been shown to be decreased in T2DM. Post-mortem investigations have reported a 30-60% decrease in β-cell mass in T2DM patients [30, 60-63] as a result of enhanced apoptosis [30, 64] associated with increased levels of caspase-3 and caspase-8 [65]. By the time of T2DM diagnosis estimations tells that about 50% of the functional β-cell mass is already lost [66]. The mechanisms involved in the apoptotic death of the β-cells is not completely known but in vitro studies have shown that free FAs can induce the apoptotic pathway [53, 67]. A deeper understanding of the mechanisms and the mediators of the apoptotic pathway will hopefully provide tools to preserve the β-cell mass and delay the progression to T2DM.

2.2.3 ER stress and ER stress induced beta-cell apoptosis

The pancreatic β-cell synthesizes massive amounts of insulin. The translation of insulin mRNA in response to glucose stimulation has been shown to account for up to 50% of the total protein synthesis in the β-cells [68, 69]. As a secretory cell, the β-cell is equipped with a highly developed endoplasmic reticulum (ER) to assist in the folding process of newly synthesized proteins [70]. In addition to its function in protein synthesis, the ER is also involved in lipid and cholesterol metabolism [71] as well as it is a major cellular Ca^{2+} store compartment [72]. ER Ca^{2+} homeostasis is critical for ER function and proper folding of newly synthesized proteins [73]. To assist the folding process, ER contains chaperons including glucose-regulated protein (GRP) 78 (also called BiP), GRP94, calnexin and calreticulin. In addition, these chaperones are part of the ER quality control system that target misfolded proteins for degradation [74]. Furthermore, ER contains protein disulphide isomerases (PDI), proteins that catalyses the protein folding process [75].

Several conditions can disturb the normal function of ER and lead to accumulation of unfolded or misfolded proteins, a condition termed ER stress. Protein overload, due to the rate of synthesis exceeds the rate of exit of proteins from ER, or alteration in the ER milieu that negatively affects the folding efficiency can lead to ER stress. To cope and survive under ER stress conditions, cells can induce a set of defence mechanisms collectively known

as the unfolded protein response (UPR) [76, 77]. UPR acts to reduce the amount of unfolded or misfolded proteins inside the ER through three major pathways: (1) translation attenuation, which prevents further accumulation of unfolded proteins; (2) up-regulation of genes encoding ER chaperones, to increase protein folding capacity; (3) degradation of misfolded proteins in the ER, called ER-Associated Degradation (ERAD) (Fig. 2). These three pathways are each controlled by a specific protein: PKR-like eIF2α kinase (PERK), activating transcription factor 6 (ATF6) and inositol requiring enzyme 1 (IRE1) respectively. Under normal conditions these three UPR transducers are attached to and held inactive by the chaperone GRP78/BiP at the ER membrane. Accumulation of misfolded proteins in the ER causes release of GRP78/BiP which results in activation of these three proteins. Upon activation, PERK forms homodimers that becomes autophosphorylated and in turn phosphorylates and inhibits eIF2α (eukaryotic translation initiation factor 2, α subunit), leading to a reduced translation rate (Fig. 2). However, the translation rate of activating transcription factor 4 (ATF4) and C/EBP homologous protein (CHOP) is increased during phosphorylation of eIF2α (p-eIF2α). Growth arrest and DNA damage-inducible protein 34 (GADD34) activates a negative feedback loop to dephosphorylate eIF2α. When ATF6 is liberated from GRP78/BiP, it is detached from the ER membrane and transported to the Golgi where it is cleaved into a functional transcription factor by site 1- and site 2 proteases. This transcription factor promotes the expression of chaperones (Fig. 2). Finally, dimerization and autophosphorylation of IRE1 activates its endoribonuclease activity that targets the X-box-binding protein 1 (XBP-1) mRNA which is alternatively spliced (XBP-1s) and translated to a functional transcription factor that, together with ATF6, promotes the expression of chaperones and also enhances the expression of proteins involved in the ERAD pathway (further described in next section) (Fig. 2) [76, 77].

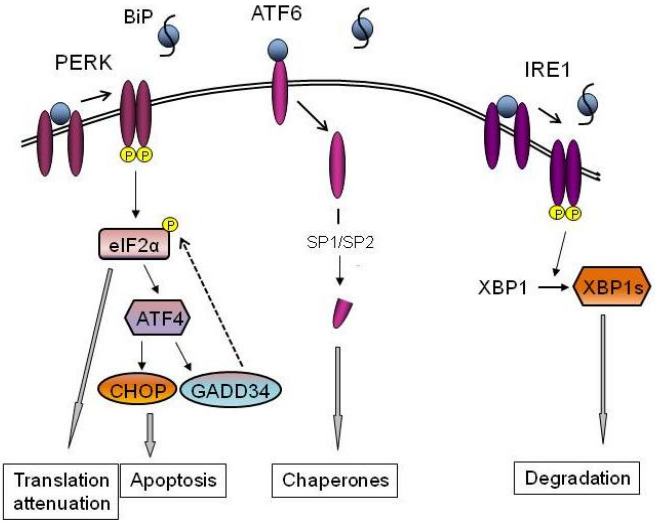

Figure 2. The main components involved in UPR. Accumulation of unfolded proteins results in GRP78/BiP release from PERK, ATF6 and IRE1 which allows these proteins to become activated. Activation of PERK promotes phosphorylation and inhibition of eF2α, leading to a reduced translation rate. However, the translation rate of ATF4 and CHOP are increased during phosphorylation of eIF2α. GADD34 activates a negative feedback loop to dephosphorylate eIF2α. Activation of IRE1 activates its endoribonuclease activity, which cleaves the mRNA of XBP-1. Upon ER stress ATF6 is released from the ER membrane and transported to the Golgi where it is cleaved to a functional transcription factor by SP1 and SP2 proteases.

If the UPR fails to relive cells from ER stress and restore ER homeostasis, the apoptosis program is activated [78, 79]. Apoptotic pathways triggered by ER stress include; transcriptional activation of the gene for CHOP by the PERK pathway of the UPR (and to some extent by ATF6), activation of caspase-12, and activation of the cJUN NH2-terminal kinase (JNK) pathway, by the IRE1 pathway of the UPR (Fig. 3). Mechanistically, CHOP has been shown to promote apoptosis through inhibition of the anti-apoptotic protein Bcl2 [78, 80, 81]. When the cell encounters ER stress, pro-apoptotic proteins from the Bcl2 family, Bak and Bax, undergo conformational changes in the ER membrane, allowing Ca^{2+} efflux from the ER. The increased concentration of Ca^{2+} in the cytoplasm activates the calcium dependent protease m-calpain which cleaves and activates procaspase-12, located in the ER membrane. Activated caspase-12 cleaves and activates procaspase-9 which leads to activation of the caspase cascade [77, 80, 82, 83]. Apoptosis via the JNK pathway starts with interaction between activated IRE1, TRAF2 (TNF

receptor-associated factor-2) and ASK1 (apoptosis signal-regulating kinase) which result in formation of the IRE1-TRAF2-ASK1 complex leading to JNK activation and cell death [84].

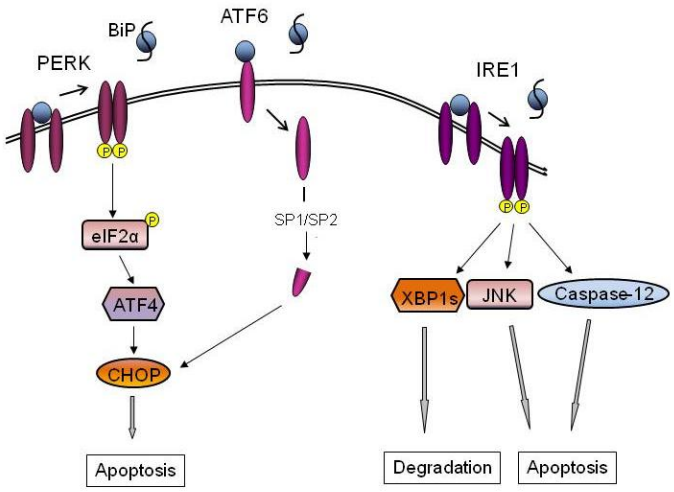

Figure 3. Apoptotic pathways induced by ER stress. Activated PERK (and to some part, activated ATF6) leads to increased expression of CHOP which inhibits the expression of Bcl2. Bak and Bax undergo conformational changes in the ER membrane allowing Ca^{2+} efflux from ER which activates the calcium dependent protease m-calpain leding to activation of procaspase-12 in the ER membrane and activation of the caspase cascade. Apoptosis via the JNK pathway starts with interaction between activated IRE1, TRAF2 and ASK1which results in formation of the IRE1-TRAF2-ASK1 and JNK activation.

Recent investigations have shown that ER stress markers are present in β-cells from type 2 diabetic patients [64, 79, 85] and in animal models of diabetes [79]. High glucose challenge of isolated islets from T2DM subjects showed increased mRNA levels of BiP/GRP78 and XBP1s compared to non-diabetic controls [64]. Investigation of pancreatic sections from T2DM donors has revealed increased protein expression of BiP/GRP78, CHOP and DNAJC3 compared to non-diabetic controls, while isolated islets from *db/db* mice revealed an up-regulation of mRNA of a variety of ER stress markers, including XBP-1s, ATF4, CHOP, Hsp90b1, BiP/GRP78 and PDIA4 [79]. An additional study has reported increased level of cytoplasmic CHOP, but also an increased nuclear translocation of CHOP in pancreas from T2DM donors compared to lean non-diabetic subjects [85]. In addition to the increased

expression of ER stress markers, expansion of ER has been found, which is a hallmark of activated UPR [64]. What is known from studies conducted in clonal insulin secreting cells and isolated pancreatic islets is that saturated FAs activates parts of the UPR followed by increased rate of apoptosis [53, 86, 87], but the triggering factor for FA-induced ER stress has not been identified. Investigations have shown that PERK and IRE1 signaling pathways of the UPR are strongly activated by palmitate [53]. ER stress has also been shown to be triggered by changes in ER Ca^{2+} handling induced by saturated FAs [53, 88] with depletion of ER Ca^{2+} and slower ER Ca^{2+} uptake. Since folding of proteins is a process dependent on Ca^{2+}, the depletion leads to misfolding of the newly synthesized proteins. Palmitate also hampers the trafficking of proteins from ER-to Golgi leading to additional accumulation of proteins in ER [89].

2.2.4 Protein degradation pathways in pancreatic beta-cells

ER stress and a compromised UPR can lead to accumulation of unfolded or misfolded proteins in the cell. Removal of the accumulated proteins is necessary before reaching toxic levels. Two main systems exist to degrade these proteins; the ERAD pathway and autophagy. A proper function of these pathways is necessary to maintain cellular homeostasis and a normal function of the cell.

2.2.4.1 ER-Associated Degradation pathway in beta-cells

The ERAD pathway for degradation of misfolded proteins is strongly connected to ER stress since this pathway is induced by the IRE1 pathway of the UPR. In addition to protein folding, the ER also compromises a quality-control system that recognizes misfolded proteins in the ERAD pathway and targets them for degradation in the ubiquitin-proteasome system, to protect the cell from ER stress [90]. In the ERAD pathway terminally misfolded proteins are translocated across the ER membrane into the cytoplasm where they are covalently bound to ubiquitin. Attachment of multiple ubiquitins gives a polyubiquitin chain, a signal for degradation by the proteasome [91]. Incubation of human and rodent islets, and MIN6 β-cells, with a proteasome inhibitor, resulted in a significant reduction in glucose-induced insulin secretion showing the importance for a functional proteasome in normal β-cell physiology [92-94]. Accumulation of polyubiqutinated proteins and low expression of the deubiquitinating enzyme ubiquitin C-terminal hydrolase L1 in islets from type 2 diabetic patients has also been reported [95]. Consistent with this, one more report showed accumulation of ubiquitinated proteins, but also a reduced

proteasomal activity and down-regulation of genes in the ubiquitin-proteasome system in islets from type 2 diabetic patients [96]. In addition to this, palmitate reduced the proteasomal activity in non-diabetic islets and INS-1E cells [96]. These reports show the importance of a functional ubiquitin-proteosomal system to protect the cell from ER stress.

2.2.4.2 Autophagy in beta-cells

Autophagy is a catabolic process that involves the degradation of proteins and organelles through the lysosomal machinery [97]. The autophagic process involves rearrangement of subcellular membranes, sequestering the proteins or organells destined for degradation, forming a double-membrane structure called autophagosome. The autophagosome fuses with the lysosome, forming the autolysosome, where the cargo is degraded by hydrolases, and the resulting macromolecules are released back to the cytosol [97]. Basal autophagy occurs at low rate in all eukaryotes to clear damage organelles, proteins and aggregates. Investigation of islets from type 2 diabetic donors has shown increased numbers of autophagosomes [98]. The accumulation of autophagosomes was suggested to be a result of defects in the lysosomal step of autophagy, indicated by decreased expression of genes involved in this, late step [98]. In addition, exposure of non-diabetic islets to FAs also led to accumulation of autophagosomes and reduced expression of genes in the late, lysosomal, step [98]. Several investigations of rodent islets and β-cells has also revealed that exposure to FFAs accumulates autophagosomes [54, 99-102]. ER stress has been suggested to be involved in the mediation of autophagy, since the synthetic chaperone 4-PBA partially abolished the induction of autophagy by FFAs in INS-1 cells [101]. *In vivo* investigations in mice with β-cell-specific disruption of the autophagy-related gene (Atg7), required for autophagy, have shown increased apoptosis and reduced proliferation, resulting in a reduction in the β-cell mass [103, 104]. Most investigations suggests that autophagy function as a protective mechanism, to restore the homeostasis during cellular stress, but it has also been suggested that autophagy may function as an executor of cell death [105].

2.3 THE METABOLIC SYNDROME

The metabolic syndrome is a medical term for a collection of abnormalities including glucose intolerance, hypertension, dyslipidemia and obesity, particularly abdominal obesity, which in combination increase the risk for T2DM and cardiovascular disease [106]. The prevalence of the metabolic syndrome is, as in the case of T2DM, increasing worldwide due to increased

energy intake, sedentary lifestyle and increasing obesity. The metabolic syndrome shares many of its abnormalities with conditions of elevated levels of the steroid hormone cortisol, including glucose intolerance [107] hypertension, abdominal obesity and dyslipidemia [108, 109], which can lead to T2DM. In addition, individuals suffering from metabolic syndrome have also been shown to have elevated levels of the cortisol [110-112]. It has therefore been suggested that the disturbed regulation of secretion and action of cortisol are involved in the progression of the metabolic syndrome [113, 114].

2.3.1 Glucocorticoids and steroid induced diabetes

Glucocorticoids (GCs), such as cortisol in humans and corticosterone in rodents, are steroid hormones produced by the adrenal cortex. These hormones exert their function in different target tissues by binding to either the GC receptor (GR) or the mineralocorticoid receptor [115]. The main physiological role of the GC hormone is to increase the level of glucose in the circulation by several mechanisms including, increased gluconeogenesis in the liver, decreased uptake of glucose into muscle and adipose tissue, breakdown of proteins and lipids to provide additional substrates for glucose production, and inhibition of insulin secretion from pancreatic β-cells [116-118].

Conditions of steroid induced diabetes [115] have been shown to develop in individuals with Cushing´s syndrome, a condition of endogenous overproduction of GCs by the adrenal cortex [119] together with the abnormalities, in the case of metabolic syndrome, described above. In addition, synthetic GCs, such as dexamethasone, that are commonly prescribed for numerous pathological conditions, including inflammation, autoimmune diseases and cancer [115, 120] have side effects of reduced insulin sensitivity, glucose intolerance and even frank diabetes [121].

Investigations performed in isolated islets and pancreatic β-cell lines have shown that exposure to GCs are toxic to these cells [122-124], but how the GCs exert their cytotoxic action in the β-cells is not fully understood. Investigations have suggested ER stress and modulation of the UPR [125], as well as p38 MAPK activation and subsequent up-regulation of thioredoxin-interacting protein [124], as possible contributors to the cytotoxic action of the GCs in pancreatic β-cells.

2.4 PROTEOMICS IN BETA-CELL RESEARCH

Proteins are the key mediators of all cellular processes. The composition of a protein is determined by its genetic code, contained within the DNA, which is replicated and transcribed into mRNA, and further translated to protein, a process called the central dogma [126] (Fig.4).

Figure 4. The central dogma of molecular biology. The central dogma involves the processes of replication, transcription and translation.

T2DM is a complex disease with altered expression of many genes and their products [12, 127]. To explore these complex changes in pancreatic β-cell, different research strategies have been used, including investigation of the genome, transcriptome and proteome. The entire set of genes in a cell is called its genome, and the large-scale study of the genome is called genomics. A genome is constant which means that all cells within an organism contain the same set of genes where the DNA is constant, and it is therefore not possible to investigate dynamic changes in response to various internal or external stimuli in the DNA sequence. However, recent progress in the field of epigenetics has revealed that modifications in, for example DNA methylation, can be induced by environmental changes [128]. In contrast to the genome, alterations of the gene expression can be measured at the mRNA level. The transcribed genes at a given time in a sample are called the transcriptome. Transcriptome analysis have generated detailed information on the gene expression in, for example β-cells [127]. However, limitations in the investigation of the transcriptome exist. These include the inability to investigate post-translational modifications that sometimes is necessary for the function of a protein [129]. Furthermore, the correlation between the levels of mRNA and its corresponding protein has been reported to be rather weak [130, 131]. These limitations can be avoided by investigation of the proteins, which are the mediators of the cellular processes.

All expressed proteins within a cell make up the proteome [132], and the research area in which proteins are studied on a large-scale is called proteomics. The human genome contains approximately 20,000 genes, each coding for one or more specific protein [133]. The corresponding number of proteins and their isoforms after post-translational modifications are

estimated to be around 1 million [134], making the proteome extremely complex. Thus, proteomics research can be a challenging task.

2.4.1 Proteomic studies performed in pancreatic islets and insulin producing clonal cells.

For the endocrine pancreas, proteomic profiling via two-dimension gel electrophoresis (2DGE) of isolated pancreatic islets has resulted in reference maps of both mouse [135] and human [136] islets, which identification 77 and 66 proteins respectively. A proteome reference map of INS-1E cells resulted in 331 unique proteins [137]. These investigations were performed by the use of 2DGE followed by identification with mass spectrometry (MS). A more recent study using LTQ-Orbitrap MS identified approximately 2000 proteins in a single islet [138]. Transcriptomic and antibody-based profiling analysis of *in vitro* isolated endocrine islets identified a set of 42 genes with elevated, compared to any tissue, in these specialized cells [139]. In addition to profiling of cells and islets, investigations of mechanisms contributing to β-cell failure have been performed both *in vivo* [140] and *in vitro* [67, 141-146]. In a study performed in human islets using surface enhanced laser desorption/ionization (SELDI) MS technique to compare the difference in the proteome between isolated islets from T2DM patients and controls, approximately 20 proteins were differentially changed in in islets from T2DM patients. Several pathways, including apoptosis, immune-response and regeneration were suggested to be activated in the T2DM islets [141]. In another study, Han et al. used the iTRAQ technique to compare islets from Zucker lean (ZL), ZF and ZDF rats, representing control, obese/prediabetes and obese/diabetes conditions, respectively [140]. A total of 54 and 58 proteins were differentially expressed in ZDF versus ZL and in ZF versus ZL, respectively. Among the potential factors mediating the progress from a prediabetic state to T2DM, impaired insulin secretion, mitochondrial dysfunction, and dysregulation of triglyceride/free FA cycling were identified by this study [140]. A proteomic approach has also been used to investigate the effects of high glucose in INS-1E cells [142]. 2DGE MS revealed 100 differentially expressed proteins compared to the control, involved in different pathways. Following high glucose, chaperone proteins were down-regulated, protein biosynthesis and ubiquitin-related proteosomal degradation were attenuated, as well as perturbations in secretion, intracellular trafficking and vesicle transport were observed [142]. Proteomics has been used for investigation of the impact of ER stress on pancreatic β-cells [143]. INS-1E cells were exposed to high and low concentrations of the ER stress inducer cyclopiazonic acid (CPA). Exposure to high concentration of CPA led to massive apoptosis and down-regulation

of proteins involved in insulin processing and chaperones, including BiP/GRP78 and PDIA3 [143].

Different proteomic approaches have also been applied to investigate the effect of palmitate in β-cells. A 2DGE-based study in INS-1E cells identified 9 proteins, related to glycolysis, ER stress and the proteasome, to be regulated by palmitate [144]. Using SELDI MS and 2DGE approaches in INS-1E cells exposed to palmitate, CPT-1 where shown to be up-regulated and calmodulin down-regulated [145]. Another 2DGE-based investigation in human islets and MIN6 cells identified carboxypeptidase E as a protein down-regulated by palmitate exposure [67]. Functional investigations showed that degradation of carboxypeptidase E contributes to ER stress and apoptosis following palmitate exposure [67]. These proteomic investigations have detected a relatively low number of proteins affected by palmitate. One possible explanation could be that palmitate mainly affects low abundant proteins which can be difficult to detect by gel-based methods. To be able to identify a larger number of proteins affected by palmitate Maris et al. reduced the complexity of the sample by subcellular prefractionation [146]. Since palmitate acts, at least in part, through ER stress they used the ER enriched fraction for their investigation, as well as full cell lysate, from INS-1E cells. In the full cell lysate, 5 proteins were found to be affected by palmitate, while the ER enriched fraction resulted in identification of 18 proteins regulated by palmitate. The investigation showed that palmitate induced β-cell dysfunction and death via ER stress, hampered insulin maturation, generation of harmful metabolites during triglyceride synthesis and altered intracellular trafficking [146].

The use of proteomics in β-cell research have provided important insights into the pathways and proteins, in various conditions connected to diabetes, which may be involved in the dysfunction of the β-cells leading to T2DM.

3 AIMS OF THE THESIS

The general aim of this thesis was to investigate the protein expression in insulin producing cells exposed to endoplasmic reticulum stress.

The specific aims were:

Paper I: To investigate the chaperone expression during an ER stress response, induced by thapsigargin in the pancreatic β-cell line INS-1E, by the use of quantitative mass spectrometry based proteomics. We also wanted investigate by which mechanisms thapsigargin decreased GRP78/BiP protein in these cells.

Paper II: To investigate the cellular protein changes in the pancreatic β-cell line INS-1E exposed to the saturated fatty acid palmitate and compare those to changes induced by the ER stress inducer thapsigargin by the use of quantitative mass spectrometry based proteomics.

Paper III: To investigate pathways involved in the cytotoxic action of glucocorticoids and the role of mitogen-activated protein kinases and protein phosphatase 5 in this process in pancreatic β-cells and islets.

4 MATERIALS AND METHODS

4.1 CULTURE AND TREATMENT OF CELLS AND ISLETS

4.1.1 INS-1E cells

Rat insulinoma, INS-1E, cells [147] (**paper I** and **II**), kindly provided by Professor Claes Wollheim, are derived from rat pancreatic β-cells and were maintained in RPMI-1640 medium (SVA, Uppsala, Sweden) containing 11 mM glucose supplemented with 10% FBS (Invitrogen, Carlsbad, CA), 10 mM HEPES (Invitrogen), 2 mM L-glutamine (SVA), 1 mM sodium pyruvate (Sigma-Aldrich, St. Louis, MO), 50 μM β-mercaptoethanol (Sigma-Aldrich) and antibiotics (6 mg/ml pencillin G and 5 mg/ml streptomycin sulfate [Invitrogen]) at 37˚C in a humidified atmosphere containing 5% CO_2. Cells were used between passages 71 and 80.

4.1.2 MIN6 cells

Mouse insulinoma, MIN6, cells [148] (**paper I** and **III**), kindly provided by Dr. Jun-ichi Miyazaki, are derived from mouse pancreatic β-cells and were maintained in DMEM medium (Invitrogen) containing 25 mM glucose supplemented with 15% FBS, 2 mM L-glutamine, 50 μM β-mercaptoethanol and antibiotics (6 mg/ml pencillin G and 5 mg/ml streptomycin sulfate) at 37˚C in a humidified atmosphere containing 5% CO_2. Cells were used between passages 22 and 30.

4.1.3 Pancreatic islets

Islets of Langerhans isolated from 3 to 6 month old male Wistar rats (**paper I**), C57Bl/6J mice (Scanbur, Sweden) (**paper I and III**), ob/ob mice (local breed, C57Bl/6J background) (**paper III**) and PP5 knockout (*Ppp5c*$^{-/-}$, local breed, C57Bl/6J background) mice (**paper III**) were used. Animals were sacrificed by exposure to CO_2 followed by decapitation. The pancreatic glands were excised and islets were isolated by collagenase digestion (Roche, Roche Diagnostics GmbH, Mannheim, Germany) and cultured in RPMI-1640 culture medium containing 11 mM glucose supplemented with 10% FBS, 2 mM L-glutamine, and antibiotics (6 mg/ml pencillin G and 5 mg/ml streptomycin sulfate) for overnight recovery at 37˚C in 5% CO_2. Animal handling was performed according to national law and approved by local ethical committee.

4.1.4 Treatment of cells and islets

Cells were plated 72 hours prior to treatment, and treatment of islets was started the day following isolation. The reagents used were: ER stress inducer thapsigargin (Sigma-Aldrich) (**paper I** and **II**), proteosome inhibitor lactacystin (Calbiochem, San Diego, CA) (**paper I**), autophagy inhibitor 3-methyladenine (3-MA) (Sigma-Aldrich) (**paper I**), and protein synthesis inhibitor cycloheximide (Sigma-Aldrich) (**paper I**). The GCs dexamethasone, prednisolone, triamcinolone, betamethasone, the GR antagonist RU486 (Sigma-Aldrich), the p38 MAPK inhibitor SB203508, and the JNK inhibitor SP600125 (Calbiochem) were all used in **paper III**. The saturated fatty acid palmitate was used to treat cells in **paper II**. Palmitate was prepared in 12.5% ethanol as 100 mM stock solutions. Prior to treatment, palmitate was allowed to complex with fatty acid-free BSA (Roche) for 30 minutes at 37 °C. Treatment of cells were performed in identical medium as the culture medium with the exception for **paper II** were the FBS concentration was 1% and the medium supplemented with 0.5% fatty acid-free BSA.

4.2 PROTEIN EXTRACTION, SDS-PAGE AND WESTERN BLOT ANALYSIS

Western blot is used to detect specific proteins in a sample. The proteins are separated according to their molecular weight by the use of sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Western blot were used to measure protein abundance in all three papers. Treated INS-1E cells and MIN6 cells were washed in PBS and lyzed on ice for 30 minutes in buffer containing 150 mM NaCl, 20 mM Tris, 0.1% SDS, 1% Triton X-100, 0.25% Na-deoxycholate, 1 mM Na3VO4, 50 mM NaF, 2 mM EDTA and Protease inhibitory cocktail (Sigma-Aldrich). After lysis, the preparation were collected and centrifuged at 14.000g for 15 minutes at 4 °C. Isolated islets were sonicated following the lysis in the buffer described above. Protein concentration was determined with Bio-Rad DC protein assay (Bio-Rad Laboratories, Hercules, CA). Equal amounts of protein were separated by 10-15 % SDS-PAGE and transferred to Immun BlotTM PVDF membranes (Bio-Rad Laboratories). Immunoblot analysis was performed using primary antibodies against GRP78/BiP, phosphorylated GR (serine 220), total GR (Abcam, Cambridge, UK), p-eIF2α, eIF2α, p-JNK, JNK, LC3B, cleaved caspase 3, phosphorylated p38 MAPK, total p38 MAPK (Cell Signaling Technology, Danvers, MA), CHOP, Chromogranin-A, cleaved spectrin α II, p53, SREBP-1, SREBP-2 , FOXO1, FOXM1, PP5, phosphorylated ASK-1 and total ASK-1 (Santa Cruz Biotechnology, Santa Cruz, CA). Horesradish peroxidase-conjugated goat anti-rabbit IgG and goat anti-mouse IgG were used as a secondary antibody (Santa Cruz Biotechnology). The protein-specific signals

were detected using Enhance Chemiluminescence (ECL) (GE-healthcare, Faifield, CT). The signals were imaged quantified using Molecular Imager ChemiDoc XRS with Quantity One Software v. 4.6.5 (Bio-Rad Laboratories). Coomassie Brilliant Blue (BioRad Laboratories) was used for staining of membranes for total protein normalization.

4.3 PROTEOMIC ANALYSIS

4.3.1 Preparation of samples

Paper I. After exposure, cells were washed, lyzed and protein concentration measured as proteins extracted for Western blot. Equal amounts of protein from each treatment condition were acetone-precipitated. The pellets were dissolved in dissolution buffer containing triethylammonium bicarbonate from the iTRAQ 8-plex kit (Applied Biosystems, Foster City, CA). Denaturant containing SDS from the iTRAQ kit was added; proteins were reduced by adding tris-(2-carboxyethyl) phosphine and alkylated by methyl methanethiosulfonate (MMTS) from the iTRAQ kit. After denaturation, reduction and alkylation, proteins were digested with trypsin (TPCK treated, Applied Biosystems). Trypsin was added, 1:50, trypsin:protein, and incubated overnight at 37 °C followed by determination of the peptide concentration.

Paper II. After exposure, cells were harvested by trypsination and washed in PBS. Cells were lysed in buffer containing 4 % SDS, 25 mM HEPES, 1 mM DTT and Protease inhibitory cocktail (Sigma-Aldrich), heated at 95°C for 5 min followed by sonication. The lysate was centrifuged for 30 mininutes at 14,000 g followed by determination of protein concentration. Equal amounts of protein (200 µg) from each treatment condition were mixed with 8 M urea (Sigma-Aldrich), 1 mM DTT, 25 mM HEPES, pH 7.6, in a centrifugation filtering unit, 10 kDa cutoff (Nanosep® Centrifugal Devices with Omega™ Membrane, 10 k), and centrifuged for 30 minutes at 14,000 g, followed by another addition of 8 M urea buffer and centrifugation. Proteins were alkylated by 50 mM iodoacetamide (Sigma-Aldrich) in 4 M urea, 25 mM HEPES, pH 7.6, for 10 minutes incubation at room temperature and centrifugation for 30 minutes at 14,000 g, followed by 2 more additions of 4 M urea and 25 mM HEPES, pH 7.6, and centrifugations for 30 minutes at 14,000 g. Trypsin (TPCK-treated, Applied Biosystems), 1:50, trypsin:protein, was added to the samples in 0.25 M urea, 25 mM HEPES and digested overnight at 37 °C. The filter units were centrifuged for 30 min, 14,000 g, followed by another centrifugation with milli-Q water and the flow-through

was collected. Peptide concentration was determined with Bio-Rad DC protein assay.

4.3.2 iTRAQ labeling

iTRAQ (Isobaric tag for relative and absolute quantification) are tags used for mass spectromey based quantification of changes in the proteome (**paper I** and **II**). iTRAQ 8-plex tags consists of a reporter group ranging from 113-121 Da (except for 120 Da) and a balancing group making the tags identical in mass and chemistry (Fig. 5).

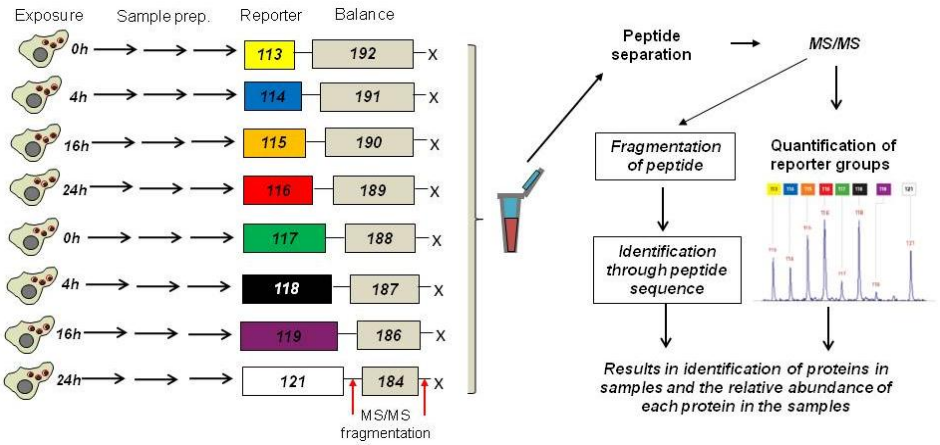

Figure 5. iTRAQ labeling for relative quantification of peptides. Cells were exposed and proteins were extracted and digested. The peptides were labeled with eight different isobaric tags and the samples were pooled. Peptides were then separated using nano-LC (**paper I**) or IEF and nano-LC (**paper II**). MALDI-MS/MS (**paper I**) or ESI-MS/MS (**paper II**) were used to identify proteins in the sample by peptide sequencing. The relative abundance of each peptide was estimated by the peak intensities of the eight different reporter groups.

iTRAQ labeling of the peptides was done according to the manufacturer's protocol (Applied Biosystems, Foster City, CA) and excess reagent and detergents were removed by strong cation exchange solid phase extraction, strata-X-C-cartridge System (Phenomenex Inc, Torrance, CA).

4.3.3 Separation of peptides

4.3.3.1 Isoelectric focusing

In isoelectric focusing (IEF), proteins or peptides are separated based on their isoelectric point (pI). This is performed by applying the proteins or peptides on an immobilized pH gradient (IPG) gel. An applied electrical current result in migration of the proteins or peptides in the IPG gel until the net charge is zero. This pre-fractionation step reduces the complexity of the sample and thus increases the proteome coverage. The iTRAQ-labeled peptides in **paper II** were pre-fractionation based on their pI on a narrow range pH 3.7-4.9 strip. Peptides were extracted from the strips in 72 fractions with MilliQ water using a prototype liquid-handling robot (GE Healthcare Bio-Science AB). Extracted peptides were dried in speed vac and dissolved in 3 % acetonitrile, 0.1% formic acid. Peptides were further separated by nano- liquid chromatography (LC) before mass spectrometry analysis.

4.3.3.2 Nano-Liquid Chromatography

Nano-Liquid Chromatography (LC) is used for separation of peptides and cleaning of the sample prior to the mass spectrometry analysis. We have used the reversed phase method which is based on the peptides interaction with a hydrophobic matrix (C_{18} column). The peptides bind to the matrix in the presence of a highly polar solvent such as water. Gradual addition of a non-polar solvent separates the peptides according to their polarity.

Paper I. Peptides were separated on an Ultimate 3000 LC system. Samples were trapped on a Symmetry C18, 100 Å, 5 µm, 180 µm x 23,5 mm trap column, and separated on a XBridge BEH130 C18, 3.5 µm, 75 µm x 150 mm nanoease column using a gradient of A (3 % ACN, 0.05 % TFA) and B (80 % ACN, 0.04 % TFA) ranging for 0 % to 45 % B in 150 min at a flow of 0.3 µl/min.

Paper II. Peptides were separated on an Agilent 1200 nano-LC system. Samples were trapped on a Zorbax 300SB-C18, and separated on a NTCC-360/100-5-153 column (Nikkyo Technos., Ltd) using a gradient of A (3 % ACN, 0.1 % FA) and B (95 % ACN, 0.1 % FA), ranging from 3 % to 40% B in 50 or 90 min with a flow of 0.4 µl/min.

4.3.4 Mass spectrometry

Mass spectrometry (MS) is a method that is used to determine the mass of a molecule. The instrument separate ions in gas phase based on their mass-to-charge ratio (*m/z*). With the use of tandem MS (MS/MS), the sequence of the molecules can be revealed by fragmentation of the sample. The three major component of a mass spectrometer are the ion source, where the peptides become ionized, the mass analyzer that determine the *m/z*, and the detector that register the number of ions at each *m/z* value. Different mass spectrometry equipment has been used in paper I and II.

4.3.4.1 MALDI-TOF/TOF

The peptides in **paper I** were analyzed with MALDI-TOF/TOF (Matrix assisted laser desorption ionization – Time of Flight/Time of Flight) (Applied Biosystems). MALDI is an ion source where the sample and a matrix, which absorb laser energy, co-crystallize on a target plate. Irradiation of the crystals results in ionization of the peptides, and the ions enter the mass analyzer. The TOF mass analyzer separate ions based on the time the ion take to move from the source to the detector. The first TOF MS isolate precursor ions and the second TOF MS analyze the fragmented ions. In our analysis, data were acquired in positive reflector mode over a mass range of 700-4000 m/z using external calibration spots with BSA digests. Mass spectra were obtained from each spot using fix laser intensity, 2000 shots per spectrum, with a uniformly random spot search pattern. A 1 kV MS/MS operating mode was used, the relative precursor mass window was set at 200 (FWHM). Collision induced dissociation (CID) fragmentation of peptides, when they collide with gas molecules, using air, was turned on, with metastable suppression enabled. Peaks with a signal/noise (S/N) ratio greater than 70 were selected for MS/MS. Up to 20 MS/MS spectra could be obtained from each spot, starting with the least intense peak and ending with the most intense peak. MS/MS acquisition of selected precursors was set to a minimum of 1500 shots per spectrum and a maximum of 2000 shots with 40 shots per sub-spectrum. The stop criteria were set to minimum of 15 peaks with a minimum S/N of 100 within the spectrum.

4.3.4.2 ESI-LTQ-Orbitrap

The peptides in **paper II** were analyzed with ESI- LTQ -Orbitrap Velos (Electrospray ionization-Linear quadropole ion trap) (Thermo Fischer Scientific, San Jose, CA). ESI generates ions in gas phase, from a sample in liquid phase, by applying a high voltage. The LTQ-orbitrap is a hybrid

instrument with two different mass analyzers. In the LTQ, ions are trapped, and peptide identification is generated by CID. In addition to the fragmentation by CID, the LTQ-Orbitrap includes high resolution ion detection with the use of higher-energy collisional dissociation (HCD). In our analysis, the instrument was operated in a data-dependent manner, selecting 5 precursors for sequential fragmentation by CID and HCD, and analyzed by the linear iontrap and orbitrap, respectively. The survey scan was performed in the Orbitrap at 30,000 resolution (profile mode) from 300-2000 *m/z,* using lock mass at *m/z* 445.120025, with a max injection time of 500 ms and AGC set to 1 x 10^6 ions. For generation of HCD fragmentation spectra, a max ion injection time of 500 ms and AGC of 5 x 10^4 were used before fragmentation at 50 % normalized collision energy. For FTMS MS2 spectra, normal mass range was used, centroiding the data at 7500 resolution. Peptides for CID were accumulated for a max ion injection time of 200 ms and AGC of 3 x 10^4, fragmented with 35 % collision energy, wideband activation on, activation q 0.25, activation time 10 ms before analysis at normal scan rate and mass range in the linear iontrap. Precursors were isolated with a width of 2 *m/z* and put on the exclusion list for 90 s. Single and unassigned charge states were rejected from precursor selection.

4.3.5 Peptide and protein identification

Paper I. Peptide identification from the MALDI-TOF/TOF data was carried out by using the Paragon algorithmin and the ProteinPilot 2.0 software package (Applied Biosystems). Default settings for a 4800 instrument were used (i.e., no manual settings for mass tolerance were given). The following parameters were selected in the analysis: iTRAQ 8-plex peptide labeled as sample type, MMTS as alkylating agent of cysteine, trypsin as digesting enzyme, 4800 as instrument, biological modifications as ID focus, and through ID as search effort. Searches were performed against the IPI database (build 3.60) limited to rat sequences. False discovery rate was estimated by searching the data against a database consisting of both forward and reversed sequences.

Paper II. All Orbitrap data were searched by Sequest-percolator under the software platform Proteome Discoverer 1.3 (Thermo Fischer Scientific) against the Uniprot rat canonical sequence protein database (27,316 entries, December 2013) using a 1 % false discovery rate cutoff. A precursor mass tolerance of 10 ppm, and product mass tolerances of 0.02 Da for HCD-FTMS and 0.8 Da for CID-ITMS were used. Further settings used were: trypsin with 1 missed cleavage; iodoacetamide on cysteine, iTRAQ 8-plex on lysine, and *N*-terminal as fixed modifications; oxidation of methionine as

variable modification. Quantification of iTRAQ 8-plex reporter ions was done by Proteome Discoverer on HCD-FTMS tandem mass spectra using an integration window tolerance of 20 ppm. Only unique peptides in the data set were used for quantitation.

4.3.6 Data distribution and extraction of regulated proteins

Paper II. Lists of differentially expressed proteins were extracted by the use of GraphPad Prism 5 and Excel. To investigate the distribution of the data and the ratio between the replicates, the data were plotted using GraphPad Prism 5. The ratio between the 0 replicate time points in the Thapsigargin data set were larger than the Palmitate data set, and Thapsigargin data were therefore used to calculate the cut off for the both data sets. The standard deviation for Thapsigargin gives a 95% confidence interval between 0.77-1.3, which is used as cut off for regulated proteins. For a protein to be defined as regulated, both replicates have to reach the cut off level. To avoid extraction of false positive proteins, proteins in the 0-time points with a ratio less than 0.77 and more than 1.3 were excluded from the list of regulated proteins.

4.3.7 Bioinformatic analysis

4.3.7.1 Gene Ontology and enrichment analysis

The Gene Ontology (GO) is a widely used biological ontology [149]. The GO consortium is an effort to standardize the annotations of genes and gene products by using a controlled and structured vocabulary of terms. One common application of the GO vocabulary is in the enrichment analysis where significantly overrepresented GO terms, in a given set of genes or gene products, are identified. GOrilla is a web-based application tool that identifies enriched GO terms in a ranked list of genes [150]. Our list of regulated proteins at each time point (the target set) were imported into the application tool and searched against all identified proteins in our data sets (the background set) for enrichments. The threshold p-value was set to $p < 10^{-3}$.

4.3.7.2 Analysis of transcription factors in Ingenuity Pathway Analysis

The web-based software from Ingenuity Systems (Ingenuity Pathway Analysis, IPA, www.ingenuity.com) was used for the search of regulated transcription factors in the Upstream Regulator tool. The upstream regulator analysis is based on prior knowledge of expected effects between

transcription regulators and their target genes in the IPA Ingenuity® Knowledge Base. This tool analyses how many known targets of each transcription factor are present in the data set and how they are regulated. This is compared to the literature to predict the activation status of a transcription factor. For each transcription factor, an overlap p-value and a z-score are computed. The overlap p-value measures if there is a statistically significant overlap between the genes in the data set compared to known targets of the transcription factor, measured by Fisher´s Exact Test. The purpose of the z-score is to measure the predicted activation status of each transcription factor.

4.4 ASSESSMENT OF APOPTOSIS AND CELL VIABILITY

The Cell Death Detection Kit ELISA^{PLUS} (Roche Diagnostics) was used to monitor apoptosis induced by the treatment (**paper I, II** and **III**). After exposure, cells were washed twice in PBS and lyzed in buffer included in the kit. Apoptosis was detected by measuring cytoplasmic DNA-histone nucleosomes, generated during apoptotic DNA fragmentation, according to the manufacturer's instructions.
Cell viability was assessed by the Cytotoxicity Detction Kit^{Plus} (Roche Diagnostics) (**paper III**). After exposure, cells were washed twice with PBS and lyzed in PBS supplemented with 1% Triton X-100. The amount of lactate dehydrogenase released after cell lysis correlates with the amount of living cells after treatment.

4.5 RNA EXTRACTION, CDNA SYNTHESIS AND QUANTITATIVE RT-PCR

Quantitative real-time Polymerase Chain reaction (PCR) was used in **paper I** and **III** for quantification of mRNA levels of GRP78/BiP, PDIA3, CHOP, Gadd34, Cdkn1a, Sgk1. After exposure, total RNA was isolated using Aurum^{TM} Total RNA Mini kit (BioRad Laboratories). Total RNA was reverse transcribed using iScript^{TM} cDNA Synthesis kit (BioRad Laboratories). The resulting cDNA was used for real-time PCR analysis using Maxima™ SYBR Green/Fluorescein qPCR Master Mix (Fermentas, Burlington, ON, Canada) (**paper I**) or SYBR Green Master mix (Thermo Scientific, Waltham, MA) (**paper III**). Amplification of the sequence of interest is achieved by using specific primers. The amplification is driven by a series of repeated temperature cycles consisting of three steps; 1) Denaturation (95 °C) where double stranded DNA dissociates through disruption of hydrogen bonds, 2) Annealing (50 °C) when the primers base pair with the template DNA, and 3) Elongation (72 °C) when the DNA polymerase synthesize a new DNA strand.

In quantitative real-time PCR the progression of the amplification is measured after each completed cycle by the use of SYBR Green which recognizes and binds to double stranded DNA. When binding, SYBR Green emits light upon excitation. The number of cycles needed to reach the threshold is dependent of the amount of mRNA in the original sample and can be used for relative comparison between samples.
Normalization was performed using the housekeeping gene Rattus norvegicus ribosomal protein L13A (Rpl13a), whose expression is not modified by thapsigargin (**paper I**) and β-actin (**paper III**).
The following formula was used for quantification: target amount = $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = (Ct_{\text{target gene}} - Ct_{\text{housekeeping gene}})_{\text{Sample}} - (Ct_{\text{target gene}} - Ct_{\text{housekeeping gene}})_{\text{Control}}$ [151].

4.6 MEASUREMENT OF XBP-1 SPLICING

For investigation of splicing of XBP-1 mRNA (**paper I**), total RNA was isolated and cDNA synthesized from INS-1E cells as described above. Rat XBP-1 cDNA was amplified using iProof Master Mix (Fermentas) and XBP-1 primers, flanking the 26 bp splicing site, which contains a PstI restriction site (CTGCAG). The PCR product was incubated with PstI (Fermentas) for 30 min at 37 °C and the digested product was separated on a 2% agarose gel and visualized using ethidium bromide. The gel shows one band at 746 bp for spliced XBP-1 and two bands at 458 bp and 314 bp for non-spliced XBP-1.

4.7 TRANSFECTION WITH SMALL INTERFERING RNA

Small interfering RNA (siRNA), used in **paper I** and **III**, is a short sequence (21-23 nucleotides) of double stranded RNA. The method is used to reduce the cellular level of a specific mRNA sequence. The sequence of the siRNA matches the sequence of the target mRNA in the cell. In **paper I**, a mixture of four different siRNA against Apg5 was used (Dharmacon RNAi Technologies, Thermo Fisher Scientific, Aalst, Belgium). 100 pmol of siRNA (25 pmol per siRNA) were introduced into INS-1E cells via nucleofection with a Nucleofector® II (Lonza, Basel, Switzerland) using Solution V and program T-20. As negative control, a non-targeting siRNA was used (Dharmacon). Exposure of cells started 48 hours post transfection. In **paper III**, siRNA targeting three different regions of mouse Ppp5c mRNA (Santa Cruz Biotechnology) were used. 100 pmol were introduced into MIN6 cells via nucleofection with a Nucleofector® II using Solution V and program G-16. As negative control, a non-targeting siRNA was used (Santa Cruz Biothechnology).

4.8 ASSESSMENT OF PROTEIN SYNTHESIS

To monitor protein synthesis (**paper I**), cells were exposed followed by starvation in a medium lacking L-methionine and L-cysteine for 1 hour. Next, Met-[35S]-label IS-103 isotope, (Biotech-IgG, Denmark) was added and cells were left for one additional hour. After labeling, cells were harvested, lyzed and radioactivity was measured in equal protein loads in a β–counter (Perkin-Elmer).

4.9 STATISTICAL ANALYSIS

Data from all used methods, except for the MS data, are presented as means ± s.e.m. Comparisons were performed by using Student's *t*-test or one-way ANOVA with Dunnett *post-hoc* test using GraphPad Prism 5. A P - value < 0.05 was deemed as statistically significant.

5 RESULTS AND DISCUSSION

T2DM is associated with a reduced function [43] and mass [50] of the pancreatic β-cells, but the exact mechanisms for the pathogenesis of the disease is still not known. As a secretory cell, the β-cell is equipped with a highly developed ER, to assist in the folding process of newly synthesized proteins [70, 75], and a proper function of this organelle is especially important in the face of an increased demand of insulin, as in the condition of insulin resistance and obesity. The general aim of this thesis was to investigate the protein expression in insulin producing cells exposed to endoplasmic reticulum stress and explore possible mechanisms involved in the progression of the reduced β-cell function and β-cell mass seen in type 2 diabetic patients.

5.1 PAPER I

Thapsigargin down-regulates protein levels of GRP78/BiP in INS-1E cells

Accumulation of misfolded proteins in the ER initiates activation of three distinct pathways of the UPR to restore the homeostasis in the ER [76, 77]. PERK and IRE1 pathways have been shown to transduce cytotoxic signals, eventually leading to apoptosis, while ATF6 pathway is considered to provide cytoprotection through up-regulation of chaperones [152, 153]. The ER contains numerous protein chaperones to assist the folding process of newly synthesized proteins [70, 75]. The ER chaperone GRP78/BiP is a central regulator of ER stress, as it controls the activation of the three transmembrane ER stress transducers. A commonly used chemical to induce ER stress and the UPR in experimental systems is the sarcoplasmic/endoplasmic Ca^{2+}-ATPas (SERCA) pump inhibitor thapsigargin, which blocks uptake of calcium ions to the ER [87, 154]. We observed that exposure of INS-1E cells to thapsigargin decreased the protein levels of GRP78/BiP instead of the expected increase. In **paper I** we investigate by which mechanisms thapsigargin decreased GRP78/BiP protein in these cells. A second aim of this paper was to investigate the chaperone expression during an ER stress response, induces by thapsigargin in INS-1E cells, by the use of quantitative mass spectrometry based proteomics.

From the positively identification and quantification of 269 unique proteins, 8 were classified as chaperones, 2 as protein disulfide isomerases (PDIs) and 2 as peptidylprolyl isomerases. The chaperones heat shock protein 90β and GRP78/BiP, and the 2 PDIs, PDIA3 and PDIA6, showed all a time-dependent

decrease after treatment with thapsigargin. GRP78/BiP, PDIA3 and PDIA6 are all localized to the ER while heat shock protein 90β is cytoplasmic. Validation of the protein expression of GRP78/BiP with Western blot corroborated proteomics data after thapsigarin treatment. In parallel with decreased protein levels of GRP78/BiP, we could see a clear induction of ER stress with enhanced phosphorylation of eIF2α (p-eIF2α), elevated protein levels of CHOP and enhanced splicing of XBP1. As expected, thapsigargin induced apoptosis, evident by elevated levels of cleaved caspase 3 and increased DNA fragmentation. The reduced protein levels of GRP78/BiP and PDIA3 are not a consequence of reduced mRNA expression since the mRNA levels of GRP78/BiP were increased and mRNA levels of PDIA3 were unaffected by the treatment with thapsigargin in the INS-1E cells.

One consequence of induced ER stress, by the PERK pathway of the UPR, is inhibition of the protein synthesis [155]. To test the hypothesis that inhibition of protein synthesis causes the reduced protein levels of GRP78/BiP, we used the protein synthesis inhibitor cycloheximide. Used concentrations of cycloheximide and thapsigargin caused a similar inhibition of protein synthesis. However, the GRP78/BiP levels were only slightly decreased after inhibition of protein synthesis and therefore we conclude that inhibition of protein synthesis by thapsigargin only explain a small part of the decreased levels of GRP78/BiP after treatment with SERCA inhibitor. We therefor investigated if the decrease of GRP78/BiP were a concequence of increased protein degradation by either the proteasome or by autophagy. Induction of ER stress enhances the ERAD pathway via the IRE1 pathway of the UPR, which directs misfolded proteins for destruction by the ubiquitin-proteasome pathway [90]. The activity of the proteasome can be inhibited by lactacystin [92]. Co-treatment of INS-1E cells with thapsigargin and lactacystin showed decreased levels of GRP78/BiP. The decrease were however smaller than by treatment with thapsigargin alone. The result indicates that proteasomal degradation contributes to parts, but not all, of the decreased levels of GRP78/BiP. Another pathway for cellular protein degradation is autophagy. Investigation showed that treatment with thapsigargin induced autophagy in INS-1E cells evident by increased mRNA levels of Atg5 and conversion of LC3B-I to LC3B-II. To elucidate the degradation of GRP78/BiP by autophagy we used the autophagy inhibitor 3-MA which inhibits the autophagosome formation by inhibiting type III Phosphatidylinositol kinases [156]. Analysis showed that the combinded treatment with thapsigargin and 3-MA gave a small decrease compared to treatment with thapsigargin alone, suggesting that degradation by autophagy can explain a small part of the decreased levels of GRP78/BiP after treatment with thapsigargin.

Since induction of chaperone expression occurs to maintain function of ER during an stress response, the data in paper I provides an explanation to why INS-1E cells are vulnerable to conditions of ER stress. Comparison of the protein levels of GRP78/BiP, and the ER stress markers CHOP and p-eIF2α, in MIN6 cells and isolated islets from mouse and rat, with the levels obtained in INS-1E cells reveals a less pronouneced induction of ER stress and not the same decrease of GRP78/BiP. The absence of decreased GRP78/BiP in MIN6 cells and islets, compared to INS-1E cells after thapsigargin treatment, may explain why INS-1E cells are sensitive to conditions of ER stress [86, 157]. Over expression of GRP78/BiP in INS-1E cells has been shown to reduce the suseptibility to thapsigargin-induced apoptosis [157], showing the importance of a functional chaperone response.

To conclude our findings in paper I, we could see a decrease of GRP78/BiP, PDIA3 and PDIA6 protein levels in INS-1E cells following thapsigargin treatment. Investigation of the mechanisms behind the decreased protein levels of GRP78/BiP indicates that this is not a concequence of reduced mRNA expression. Rather, the reduction results from the combined effect of reduced protein synthesis and enhanced proteasomal degradation, and possibly also degradation via autophagy. Induction of ER stress by thapsigargin leads to lower protein levels of GRP78/BiP, PDIA3 and PDIA6 in INS-1E cells which may contribute to the suceptibility to ER stress in this β-cell model.

5.2 PAPER II

Metabolic Master Transcription Factors affected by treatment with Palmitate and Thapsigargin - A Proteomic Investigation of the Insulin-Producing Cell Line INS-1E

T2DM is closely associated with obesity [158], where affected individuals display alterations in the circulating lipid profile [18, 19]. There is evidence that the elevated systemic levels of FAs are detrimental to pancreatic β-cells which may contribute to the progression of β-cell failure via both impaired function and a reduction of the β-cell mass seen in T2DM patients [21, 22, 49, 50]. Investigations indicate that the reduced β-cell mass may be a result of increased apoptosis [30, 51, 65], but the mechanism is not completely known. There is evidence that saturated FAs can induce apoptosis [51, 52, 159], as well as ER stress [70, 160]. This organelle is sensitive to alteration in homeostasis, and an imbalance between protein load and folding capacity can result in ER stress. In **paper II** we investigated the cellular protein

changes that occurred in the pancreatic β-cell line INS-1E exposed to the saturated FA palmitate and compare those to changes induced by the ER stress inducer thapsigargin. To this end we used quantitative mass spectrometry based proteomics.

Before the proteomic experiment, we established conditions where palmitate and thapsigargin induced the same degree of apoptosis, evident by DNA fragmentation (Fig.6). Western blot data confirm that the used concentrations of palmitate and thapsigargin induce ER stress, by measuring CHOP and phosphorylation of eIF2α, although thapsigargin was shown to be a more potent inducer of ER stress than palmitate at these concentrations (Fig.6).

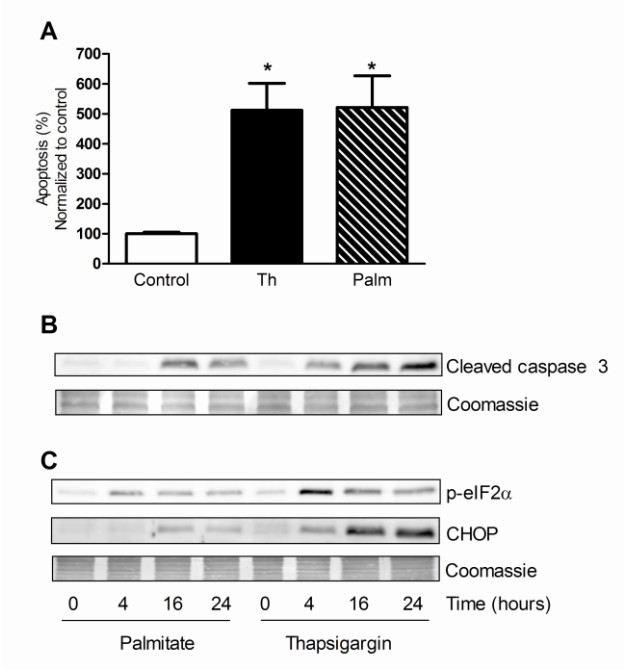

Figure 6. Treatment with thapsigargin or palmitate induces apoptosis and ER stress in INS-1E cells. Cells were exposed to 200 nM thapsigargin or 0.4 mM palmitate for indicated time points. (A) Apoptosis was evaluated after 16 hours of treatment by using a cell death detection ELISA kit, measuring the levels of cytoplasmic oligonucleosomes. The data were normalized to the control condition. Bars represent mean ±SEM, n = 9. *P<0.05. (B, C) Blots showing protein levels of cleaved caspase 3, CHOP and phosphorylation status of eIF2α.

In previous investigations of proteome changes in β-cells following ER stress, induced by cyclopiazonic acid, only a limited number, 34-101, of

differentially expressed proteins were identified [143], while exposure to palmitate resulted in detection of 5 to 19 differentially regulated proteins [67, 144, 146]. To increase the number of detected proteins, we reduced the complexity of the sample by narrow range isoelectric focusing [161] before nano-LC-MS/MS analysis. The time series data from thapsigargin- and palmitate data sets resulted in identification of 7,786 proteins of which 6,117 were quantified in both data sets. In total, 1,088 proteins were considered to be regulated by any of the treatments in at least one of the time points examined. 1,000 proteins were up- or down-regulated by thapsigargin and 227 were affected by palmitate. Thus, during the experimental setting employed in this study, the proteomic changes induced by thapsigargin exceeded those induced by palmitate.

Among the regulated proteins by thapsigargin and palmitate, a significant portion was localized to ER, in concordance with induction of UPR. Of all 1,088 regulated proteins, 136 were GO annotated to ER. Of these proteins, 108 were only affected by treatment with thapsigargin. Palmitate treatment affected 22 ER proteins, of which 15 also were affected by thapsigargin (12 down-regulated and 3 up-regulated). Of the 7 proteins specifically affected by palmitate, 4 were down-regulated and 3 up-regulated. ER-localized proteins were enriched at all tested time points after treatment with thapsigargin, while ER proteins were enriched only after 24 hours treatment with palmitate. This corroborated the findings that thapsigargin induced a more rapid effect on ER compared to palmitate, as indicated by the stronger induction of ER stress seen in Western blot data.

In our search for potential biological processes shared by thapsigargin and palmitate treatment in our data sets, we searched for regulated transcription factors, using Ingenuity Pathway Analysis (IPA) software. In this analysis we found that both thapsigargin and palmitate inhibited sterol regulatory element-binding protein (SREBP) -1 and SREBP-2 which are regulators of genes involved in FA- and cholesterol synthesis [162]. Furthermore, the transcription factor forkhead box protein O1 (FOXO1), involved in regulation of the cell cycle, apoptosis, autophagy and metabolism [163], as well as forkhead box protein M1 (FOXM1), a regulator of the expression of cell cycle genes essential for DNA replication and mitosis [164], were both predicted to be inhibited by thapsigargin and palmitate.

The rapid decrease of SREBP-2 by thapsigargin, seen in proteomics data, was validated by Western blot. As predicted by IPA, Western blot data confirms the inhibition of SREBP-2 activity by thapsigargin, while treatment

with palmitate induces an early, transient activation. Western blot data also show decreased levels of SREBP-1 precursor by thapsigargin while the levels were increased after palmitate treatment. None of the treatments activated SREBP-1, confirming the predicted activation status by IPA. It has been reported that treatment of INS-1E cells not activates SREBP-1 in response to FAs [165]. In addition, inhibition of transcription factors and pathways involved in lipid and cholesterol metabolism have been reported in liver after induction of ER stress with tunicamycin [166, 167]. Palmitate was also reported to decrease SREBP-1 levels in hepatocytes [168]. On the other hand, SREBP-1 has been reported to be increased in liver and islets of diabetic animals [169]. One suggestion for the increased SREBP-1 levels in the diabetic animals is that the hyperglycemia, rather than the FAs, causes the increase since glucose has been shown to activate this transcription factor [165].

In **paper II** we identified 6,117 unique proteins expressed in INS-1E cells, making this study one of the most detailed proteomic studies of insulin producing cells. Treatment with the saturated FA palmitate and the SERCA pump inhibitor thapsigargin shared a profound effect on UPR and ER localized proteins. The results also show that palmitate and thapsigargin down-regulates proteins involved in biosynthesis of cholesterol and FAs, as well as proteins involved in cell cycle regulation. The treatments not only share the induction of ER stress but also share an effect on the lipid handling in the cell.

5.3 PAPER III

Mitogen-activated protein kinases and protein phosphatase 5 mediate glucocorticoid-induced cytotoxicity in pancreatic islets and β-cells.

Glucocorticoid (GC) excess is associated with glucose intolerance and diabetes by mechanisms including, increased gluconeogenesis in the liver, decreased uptake of glucose into muscle and adipose tissue, breakdown of muscle and lipids to provide additional substrates for glucose production, and inhibition of insulin secretion from pancreatic β-cells [116-118]. The direct effect of GCs on pancreatic β-cells and islets is difficult to study during *in vivo* conditions since the metabolic actions of GCs interfere with the direct effects induced by GCs. Investigations in isolated islets and pancreatic β-cell lines have shown that exposure to GCs are toxic to these cells [122-124] but how the GCs exert their cytotoxic action in the β-cells is not fully understood. Investigations have shown that dysfunction of ER homeostasis [125] and MAPK activation might contribute to the cytotoxic effect in β-

cells, induced by GCs [124]. The action of protein kinases are countered by protein phosphatases, and the phosphatase activity of the GC receptor (GR) have been shown to be catalyzed by protein phosphatase 5 (PP5) that binds to GR via interaction with heat-shock protein 90 [170, 171]. In **paper III** we investigate pathways involved in the cytotoxic action of GCs and the role of MAPKs and PP5 in this process in pancreatic β-cells and islets.

As expected, exposure of MIN6 cells to different synthetic GCs reduced the viability of the cells, and exposure of MIN6 cells and isolated mouse islets to dexamethasone induced apoptosis, evident by DNA fragmentation. The cytotoxic effects induced by the GCs were reversed by the GR antagonist RU486. To gain insight into the cytotoxic action induced by GCs we investigated ER stress and the PERK pathway of the UPR, by measuring p-eIF2α, and mRNA expression of the pro-apoptotic genes CHOP and GADD34, in MIN6 cells exposed to dexamethasone. The result showed a reduced phosphorylation of eIF2α, as previously shown [125], as well as decreased mRNA levels of CHOP and GADD34, indicating a reduced activity of this pathway of the UPR. Activation of the PERK pathway of the UPR acts to alleviate the ER stress by reducing the protein translation, and thereby reduce the influx of proteins into ER [77]. Our result indicate a compromised activity of the UPR following treatment with dexamethasone, which may lead to a reduced capacity to restore the homeostasis in the ER. Failure to induce UPR has been linked to abnormalities in β-cell gene expression and progression towards diabetes in *db/db* mice [172].

To further explore the cytotoxic effects inducd by dexamethasone in MIN6 cells, we investigated the MAPK siganaling pathway. The MAPK proteins p38 and JNK are known to regulate cell differentiation and apoptosis and have been shown to be activated by various forms of environmental stress and inflammation [173]. We observed an increased phosphorylation of both p38 and JNK, following treatment with dexamethasone, an effect that was reversed by RU486. Further investigation showed that p38 and JNK, as well ASK-1, an upstream kinase activating both p38 and JNK [173], were phosphorylated by dexamethasone in an time-dependent manner. Pharmacological inhibition of p38 MAPK, by SB203580 in MIN6 cells, attenuated dexamethasone-induced DNA fragmentation and impoved the viability of the cells. In addition, dexamethasone-induced induction of cleaved caspase 3, in isolated islets from mice, was prevented by SB203580 alsmost to the same extent as with RU486. It has been demonstrated that p38 MAPK phosphorylates GR at serine 220 (corresponding to serine 211 in human) which stimulates the activity of the GR [174]. We could see that exposure of MIN6 cells to dexamethasone induced phosphorylation att

serine 220, an effect that to some extent was reversed by SB203580, suggesting a contribution of p38 MAPK phosphorylation at serine 220 of the GR, in the cytotoxcicity induced by dexamethasone. In contrast to the protective effect by inhibition of p38 MAPK, the JNK inhibitor SP600125 agumented the dexamethasone-induced apoptosis. It has been demonstrated that JNK is responsible for phosphorylation of serine 234 of the GR which leads to a loss of GR action [175]. We could also show that the effect of JNK inhibition is dependent on a functional GR since inhibition of GR abolished the agumenting effect off SP600125 on dexamethasone-induced apoptosis.

The activity of GR is not only regulated by kinases, but also by phosphatases. The phosphatase PP5 has been shown to decrease phosphorylation of GR at serine 220/211, resulting in a reduced sensitivity to GC treatment in airway smooth muscle cells [176]. In addition, suppression of PP5 has shown to increas the transcriptional activity of GR [171], supporting a role for PP5 as a regulator of GR activity. We show that MIN6 cells and islets with reduced levels of PP5 display increased suseptibility to dexamethasone-induced apoptosis. In addition, the GC-induced phosphorylation of p38 MAPK is agumented in cells and islets lacing PP5, suggesting that PP5 function as a mediator of β-cell protection against GC-induced cytotoxicity.

In conclusion, our data show that dexamethasone activates ASK-1 and its downstream targets p38 MAPK and JNK in β-cells, which work in opposite to regulate the cytotoxic effects induced by GCs. The data also suggests that PP5 play a protective role, since reduced PP5 levels in MIN6 cells and islets makes the cells sensitized to the toxic effects induced by GCs. Our results also indicate a compromised activity of the UPR, which may lead to a reduced capacity to restore the homeostasis in the ER.

6 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

6.1 CONCLUDING REMARKS

The prevalence of T2DM is increasing rapidly worldwide as a result of the obesity epidemic, and is associated with severe and long-term complications of affected individuals, as well as an enormous economic burden to health care systems. It is therefore of great importance to elucidate the mechanisms contributing to the progression of the disease. There is evidence that the elevated levels of FAs are detrimental to pancreatic β-cells which may contribute to the progression of β-cell failure via both impaired function and a reduction of the β-cell mass seen in T2DM patients. Saturated FAs can induce apoptosis, as well as ER stress. As a secretory cell, the pancreatic β-cell is equipped with a highly developed ER to assist in the folding process of newly synthesized proteins, but this cell organelle is sensitive to alteration in homeostasis, and an imbalance between protein load and folding capacity can result in ER stress. This thesis aimed to investigate the protein expression in insulin producing cells exposed to ER stress. In **paper I**, levels of the ER localized protein GRP78/BiP, PDIA3 and PDIA6 were decrased by thapsigargin in INS-1E cells. The decrease involved a combination of reduced protein synthesis and enhanced degradation by both proteasome and autophagy. The data in paper I provides an explanation to why INS-1E cells are vulnerable to conditions of ER stress. In **paper II,** we identified 7,786 proteins by the use of proteomic in INS-1E cells exposed to palmitate and thapsigargin. 1,000 proteins were regulated by thapsigargin and 227 by palmitate. Bioinformatic analysis revealed a set of transcription factors predicted to be regulated in the same manner by the thapsigargin and palmitate. This paper shows that the treatments not only share the induction of ER stress but also share an effect on the lipid handling in the cell by affecting several metabolic master transcription factors.

In addition to increased prevalence of T2DM and obesity, the prevalence of metabolic syndrome is also increasing. This condition is associated with increased risk of developing T2DM. In addition, individuals suffering from metabolic syndrome have been shown to have elevated levels of cortisol and therefore, in **paper III** we wanted to investigate pathways involved in the cytotoxic action of GCs since excess of GCs is associated with glucose intolerance and diabetes. The data show that GC activates p38 MAPK and JNK in β-cells, which work in opposite to regulate the cytotoxic effects

induced by GCs. The data also suggests that PP5 play a protective role, since reduced PP5 levels in MIN6 cells and islets makes the cells sensitized to the toxic effects induced by GCs. Our result also indicate a GC-induced compromised activity of the UPR, which may lead to a reduced capacity to restore the homeostasis in the ER.

6.2 FUTURE PERSPECTIVES

The time series data from the proteomics analysis in **paper II** resulted in identification of 7,786 proteins in two independent data sets. 1,000 proteins after treatment with thapsigargin and 227 proteins after treatment with palmitate were found to be either up- or down-regulated in at least one time point. It would be of interest to further investigate the proteins affected by the treatments. Tools such as siRNA mediated knock down of mRNA expression, as well as vectors used for induced over expression would certainly be important tools to elucidate functional consequences of these proteins. It is possible to investigate which pathways and networks the regulated proteins are involved in. Categorization by cellular localization of all regulated proteins can also be done to investigate where in the cell, apart from the ER, the regulated proteins are localized. Categorization of biological function and cellular processes of the regulated proteins would also be possible. Another interesting aspect to analyze in the data sets are proteins affected at the early time point and compare those to proteins affected at a later time point.

A deeper investigation of the proteins affected by the treatments would be of great value, since both ER stress and FAs are implicated in the dysfunction of β-cells in the progression to T2DM.

7 SVENSK SAMMANFATTINING

Antalet människor som drabbas av typ 2 diabetes ökar i hela världen. Denna ökning går hand i hand med en ökad förekomst av övervikt och fetma. Vår västerländska livsstil med ett högt kaloriintag tillsammans med att vi rör oss för lite är en farlig kombination. Typ 2 diabetes kännetecknas av att den drabbade inte kan kontrollera sin blodsockernivå, vilket på sikt kan leda till komplikationer. Nivåerna av socker i blodet registreras av β-celler som finns i bukspottkörteln. De svarar på en ökad sockernivå genom att producera och utsöndra insulin till blodet. Insulinet behövs för att muskler och fett ska kunna ta upp socker. Detta leder till att nivåerna av socker sjunker till det normala i blodet. Hos en typ 2 diabetiker klarar inte β-cellerna av att utsöndra tillräckligt med insulin för att sänka blodsockernivåerna.

Studier har visat att typ 2 diabetiker har en försämrad funktion hos β-cellerna, men även en ökad β-cellsdöd. Vad som orsakar denna försämring är inte helt klarlagd. Individer som lider av fetma har ökade nivåer av fettsyror i blodet och man har sett att fettsyror kan leda till försämrad funktion och död hos β-cellerna. Man har även sett att fettsyror kan leda till sk endoplasmatisk retikulum stress (ER stress), något som uppstår då cellen inte förmår att bearbeta (vecka) nybildade proteiner, som t.ex. insulin, så att de antar sin rätta form. Cellen försöker då skydda sig genom att öka tre signaleringsvägar som tillsammans kallas ER stressrespons. Om detta försök misslyckas aktiverar cellen istället siganleringsvägar för celldöd.

Forskningen som ligger till grund för denna avhandling har undersökt proteinnivåer hos insulinproducerande celler som exponerats för ER stress. I **studie I** använde vi oss av en teknik som kallas proteomik. Denna teknik kan användas för att studera i princip alla proteiner i cellen på en och samma gång. Syftet med proteomikundersökningen var att undersöka proteiner som hjälper till i veckningsprocessen. Insulinproducerande celler utsattes för en substans som inducerar ER stress innan undersökningen. Undersökningen visade att tre av de identifierad proteinerna som hjälper till i veckningsprocessen var minskade när cellen utsattes för ER stress. Fortsatta undersökningar av ett av dessa proteiner visade att de minskade nivåerna beror på att att cellen minskar syntesen av proteinet, i kombination med en ökad nedbrytning genom två olika processer. Studien visar att en fungerande veckningsprocess krävs för att cellen ska överleva.

Även i **studie II** använde vi proteomiktekniken. Celler exponerade för den mättade fettsyra palmitat jämfördes med celler exponerade för den ER

stressinducerande substansen. I denna studie identifierade vi 7,786 proteiner. Av dessa var 227 ökade eller minskade efter exponering för fettsyran, medan 1,000 var ökade eller minskade efter exponering för den ER stressinducerande substansen. Fortsatta analyser visade en grupp regleringsproteiner som påverkas på samma sätt av de båda behandlingarna. Denna studie visde att både den mättade fettsyran och ER stressinduceraren orsakar ER stress i cellen, samtidigt som båda även påverkar fettmetabolismen i cellen genom att påverka regleringsproteinerna på ett likartat sätt.

Samtidigt som typ 2 diabetes och fetma ökar i världen så ökar även det ”metabola syndromet” vilket är ett samlingsnamn för olika riskfaktorer för ämnesomsättningssjukdom. Det metabola syndromet är förknippat med en ökad risk att drabbas av hjärt-kärlsjukdomar och typ 2 diabetes. Det har visats att individer med metabola syndromet har ökade nivåer av kortisol i blodet. Kortisol tillhör en grupp steroidhormoner som kallas glukokortikoider. En rad läkemedel är baserade på glukokortikoider och de används t.ex för att dämpa allergiska reaktioner. En biverkan hos denna typ av läkemedel är symptom förknippade med metabola syndromet och även utveckling av diabetes. I **studie III** kunde vi se att β-celler exponerade för glukokortikoider dog. Vi ville därför undersöka vilka signaleringsvägar som var förknippade med de negativa effekter på cellen efter glukokortikoidbehandling. Vi såg att ett protein, proteinfosfatas 5, var viktig för att reglera effekten av glukokortikoider. Vi kunde visa att om detta protein saknas blir cellerna extra känslig för de negativa effekterna som glukokortikoiderna ger.

Studier på insulinproducerande β-celler är nödvändig för att få en större förståelse för de mekanismer och faktorer som reglerar de negativa effekterna på dessa celler. Detta kommer förhoppningsvis i framtiden leda till verktyg för att bevara funktionen och mängden av β-cellerna, och på så sätt hindra eller fördröja utvecklingen av typ 2 diabetes.

8 ACKNOWLEDGEMENTS

Då var det slutligen dags att tacka alla som på ett eller annat sätt bidragit till denna avhandling. Det hade inte gått utan er.

Först och främst vill jag tacka min huvudhandledare **Henrik Ortsäter**. Denna resa har varit lång och allt annat än rak, men tack vare ditt stöd är nu målet nära. Du är inte bara en duktig forskare, du är även en inspirerande handledare och jag är väldigt tacksam över att jag fått ha just dig som handledare. Tack för all hjälp jag fått under dessa år.

Jag vill också tacka min bihandledare **Henrik Johansson**. Det har alltid varit både trevligt och lärorikt att få åka på möten på Scilife Lab. Tack för allt du lärt mig om proteomik.

Ett stort tack till alla underbara vänner och doktorandkollegor på KI SÖS. Det har varit väldigt inspirerande att få jobba med er, men också väldigt rolig. Underbara **Lotta**, jag är så glad över att fått lära känna dig. Tack för alla långa diskussioner vi haft om allt mellan himmel och jord, och tack för allt kul vi hunnit med. Med dig kan man verkligen vara sig själv. **Linnéa**, du är verkligen en genomsnäll person som sprudlar av energi. Tack för alla diskussioner genom åren och tack för att jag har någon att prata USA-resor med. **Shiva**, du är en av de mest rara personer jag känner. Tack för att jag fått lära känna dig och tack för att du delat med dig av dina funderingar. Du har förgyllt många av mina dagar. **Camilla**, du är en underbar person som jag är glad över att jag fått lära känna och jag är oerhört imponerad över hur mycket du hinner med. Tack för allt stöd under dessa år. **Petra**, du kan konsten att muntra upp människor med ditt leende. Du är även den som är bäst på att dra skämt. Tack för att jag fått lära känna dig. **Nina**, jag är glad över att jag fått lära känna dig och få möjlighet att jobba med dig. Kul att du äntligen är tillbaka. **Anna**, vi hann inte jobba så länge tillsammans, men jag är glad för den tiden vi hann med. Vi får se till att träffas ibland så du kan berätta om alla dina äventyr. **Özlem**, tack för tiden tillsammans på KI SÖS. **Åke**, tack för all kunskap du delat med dig av, och **Thomas**, tack för all input på våra gruppmöten.
I would also like to thank **Cesare**, **Irakli** and **Qimin** for your friendship and for contributing to the scientific environment at KI SÖS.

Jag vill även tacka **Janne Lehtiö** och hans grupp. Jag är glad över att jag fått möjlighet att genomföra mina proteomikstudier hos er på Scilife Lab.

Tack till **Per Tornvall**, nuvarande prefekt på Institutionen för klinisk forskning och utbildning, Södersjukhuset. Stort tack även till tidigare prefekter, **Maaret Castrén** och **Göran Elind**er. Stort tack även till all annan personal på KI SÖS, speciellt tack till: **Christina Hadders Medin**, **Emelie Berglund** och **Jeanette Öhrman**. Jag vill även tacka studierektor **Christer Svensén** för all hjälp under doktorandutbildningen.

Tack till Forskningscentrums nuvarande och tidigare chefer, **Jeannette Lundblad-Magnusson** och **Michael Eberhardson**. Extra tack till **Jeannette** som kan lösa de flesta problem på labbet, alltid med ett leende på läpparna. **Mohamed Eweida**, du är en klippa på labb. Förutom att du är väldigt kunnig, så är du även väldigt snäll och omtänksam. Tack för att du alltid tar dig tid att prata med mig. Tack även till **Carina** och alla andra på plan 5.

Tack alla lunchvänner på forskningscentrum på plan 3. **Elizabeth**, tack för all hjälp med knäproblem. Tack även till **Viveca**, **Axel**, **Sara**, **Helen**, **Tina**, **Lina, Hasse, Katarina**, **Kerstin** och alla ni andra.

Ett stort tack till min externa mentor **Nina Rökeus**.

Tack till hela släkten, både min egen och den jag är ingift i. **Mamma** och **Stig**, **Pappa** och **Ingela**, jag hoppas att jag kommer få mer tid över för att komma och hälsa på nu. Tack lillasyster **Veronica** och lillebror **Christoffer** med familjer. **Viveca**, jag kommer aldrig glömma dig. **Anna**, **Daniel** och **Jonatan**, jag hoppas att det kommer bli mer tid över för att träffas när jag disputerat. **Johan**, **Lisa** och era underbara barn **Hugo** och **Frans**. **Malin**, **Mats** och er duktiga hockeykille **Axel**. **Karin**, **Olle** och **Sara**. Det är alltid så härligt när vi träffas.

Gunilla, en bättre svärmor än du finns inte. Det finns inte någon annan som kan få mig att känna mig så avslappnad som du. Ser fram emot många middagar med ett glas vin och mycket prat. Tack för all hjälp vi har fått under alla år. Ett stort tack även till min svärfar **Janne** som tyvärr inte fick vara med hela vägen till min disputation. Vi saknar dig.

Mormor och **morfar**, ni kommer alltid ha en speciell plats i mitt hjärta.

Mest av allt vill jag tacka min stora kärlek **Mattias** och våra underbara barn **Felix**, **Vendela** och **Filip**. Tack **Mattias** för att du alltid finns där för mig och tack för att du har stått ut med mig under denna intensiva slutfas ☺.
De 21 åren tillsammans med dig har vart de bästa i mitt liv.
Tack **Felix**, **Vendela** och **Filip** för att ni fått mig att förstå vad som är viktigast i livet. Ni är allt jag någonsin önskat mig. Jag ser fram emot att fortsätta våra äventyr tillsammans bland indianer, cowboys och mycket, mycket mer.

Tyresö, januari 2015

9 REFERENCES

1. Poretsky, L. and SpringerLink, *Principles of Diabetes Mellitus*. 2010, Boston, MA: Springer US.

2. *IDF Diabetes Atlas*. 2013; Available from: http://www.idf.org/diabetesatlas.

3. Zimmet, P.Z., et al., *Diabetes: a 21st century challenge.* Lancet Diabetes Endocrinol, 2014. **2**(1): p. 56-64.

4. *Diagnosis and classification of diabetes mellitus.* Diabetes Care, 2014. **37 Suppl 1**: p. S81-90.

5. *Report of a WHO/IDF Consultation, Definition and Diagnosis of Diabetes Mellitus and Intermediate Hyperglycaemia, World Health Organization.* 2006.

6. Schwarz, P.E., et al., *Nonpharmacological interventions for the prevention of type 2 diabetes mellitus.* Nat Rev Endocrinol, 2012. **8**(6): p. 363-73.

7. Halban, P.A., et al., *beta-cell Failure in Type 2 Diabetes: Postulated Mechanisms and Prospects for Prevention and Treatment.* J Clin Endocrinol Metab, 2014: p. jc20141425.

8. Kahn, S.E., M.E. Cooper, and S. Del Prato, *Pathophysiology and treatment of type 2 diabetes: perspectives on the past, present, and future.* Lancet, 2014. **383**(9922): p. 1068-83.

9. Morris, A.P., et al., *Large-scale association analysis provides insights into the genetic architecture and pathophysiology of type 2 diabetes.* Nat Genet, 2012. **44**(9): p. 981-90.

10. Ng, H.J. and A.L. Gloyn, *Bridging the gap between genetic associations and molecular mechanisms for type 2 diabetes.* Curr Diab Rep, 2013. **13**(6): p. 778-85.

11. Herder, C. and M. Roden, *Genetics of type 2 diabetes: pathophysiologic and clinical relevance.* Eur J Clin Invest, 2011. **41**(6): p. 679-92.

12. Brunetti, A., E. Chiefari, and D. Foti, *Recent advances in the molecular genetics of type 2 diabetes mellitus.* World J Diabetes, 2014. **5**(2): p. 128-40.

13. Astrup, A. and N. Finer, *Redefining type 2 diabetes: 'diabesity' or 'obesity dependent diabetes mellitus'?* Obes Rev, 2000. **1**(2): p. 57-9.

14. Kahn, S.E., R.L. Hull, and K.M. Utzschneider, *Mechanisms linking obesity to insulin resistance and type 2 diabetes.* Nature, 2006. **444**(7121): p. 840-6.

15. Kahn, B.B. and J.S. Flier, *Obesity and insulin resistance.* J Clin Invest, 2000. **106**(4): p. 473-81.

16. Reinehr, T., *Type 2 diabetes mellitus in children and adolescents.* World J Diabetes, 2013. **4**(6): p. 270-81.

17. Pulgaron, E.R. and A.M. Delamater, *Obesity and type 2 diabetes in children: epidemiology and treatment.* Curr Diab Rep, 2014. **14**(8): p. 508.

18. Yi, L., et al., *Simultaneously quantitative measurement of comprehensive profiles of esterified and non-esterified fatty acid in plasma of type 2 diabetic patients.* Chem Phys Lipids, 2007. **150**(2): p. 204-16.

19. Rhodes, C.J., *Type 2 diabetes-a matter of beta-cell life and death?* Science, 2005. **307**(5708): p. 380-4.

20. Bergman, R.N. and M. Ader, *Free fatty acids and pathogenesis of type 2 diabetes mellitus.* Trends Endocrinol Metab, 2000. **11**(9): p. 351-6.

21. Kashyap, S., et al., *A sustained increase in plasma free fatty acids impairs insulin secretion in nondiabetic subjects genetically predisposed to develop type 2 diabetes.* Diabetes, 2003. **52**(10): p. 2461-74.

22. Salgin, B., et al., *Higher fasting plasma free fatty acid levels are associated with lower insulin secretion in children and adults and a higher incidence of type 2 diabetes.* J Clin Endocrinol Metab, 2012. **97**(9): p. 3302-9.

23. Cabrera, O., et al., *The unique cytoarchitecture of human pancreatic islets has implications for islet cell function.* Proc Natl Acad Sci U S A, 2006. **103**(7): p. 2334-9.

24. Bonner-Weir, S. and T.D. O'Brien, *Islets in type 2 diabetes: in honor of Dr. Robert C. Turner.* Diabetes, 2008. **57**(11): p. 2899-904.

25. In't Veld, P. and M. Marichal, *Microscopic anatomy of the human islet of Langerhans.* Adv Exp Med Biol, 2010. **654**: p. 1-19.

26. Edlund, H., *Pancreatic organogenesis--developmental mechanisms and implications for therapy.* Nat Rev Genet, 2002. **3**(7): p. 524-32.

27. Bouwens, L., W.G. Lu, and R. De Krijger, *Proliferation and differentiation in the human fetal endocrine pancreas.* Diabetologia, 1997. **40**(4): p. 398-404.

28. Gregg, B.E., et al., *Formation of a human beta-cell population within pancreatic islets is set early in life.* J Clin Endocrinol Metab, 2012. **97**(9): p. 3197-206.

29. Meier, J.J., et al., *Beta-cell replication is the primary mechanism subserving the postnatal expansion of beta-cell mass in humans.* Diabetes, 2008. **57**(6): p. 1584-94.

30. Butler, A.E., et al., *Beta-cell deficit and increased beta-cell apoptosis in humans with type 2 diabetes.* Diabetes, 2003. **52**(1): p. 102-10.

31. Jensen, M.V., et al., *Metabolic cycling in control of glucose-stimulated insulin secretion.* Am J Physiol Endocrinol Metab, 2008. **295**(6): p. E1287-97.

32. Moore, K.A. and J. Hollien, *The unfolded protein response in secretory cell function.* Annu Rev Genet, 2012. **46**: p. 165-83.

33. Henquin, J.C., *The dual control of insulin secretion by glucose involves triggering and amplifying pathways in beta-cells.* Diabetes Res Clin Pract, 2011. **93 Suppl 1**: p. S27-31.

34. De Vos, A., et al., *Human and rat beta cells differ in glucose transporter but not in glucokinase gene expression.* J Clin Invest, 1995. **96**(5): p. 2489-95.

35. McCulloch, L.J., et al., *GLUT2 (SLC2A2) is not the principal glucose transporter in human pancreatic beta cells: implications for understanding genetic association signals at this locus.* Mol Genet Metab, 2011. **104**(4): p. 648-53.

36. Newsholme, P., et al., *Nutrient regulation of insulin secretion and action.* J Endocrinol, 2014. **221**(3): p. R105-20.

37. Komatsu, M., et al., *Glucose-stimulated insulin secretion: A newer perspective.* J Diabetes Investig, 2013. **4**(6): p. 511-6.

38. Olofsson, C.S., et al., *Fast insulin secretion reflects exocytosis of docked granules in mouse pancreatic B-cells.* Pflugers Arch, 2002. **444**(1-2): p. 43-51.

39. Sugden, M.C. and M.J. Holness, *The pyruvate carboxylase-pyruvate dehydrogenase axis in islet pyruvate metabolism: Going round in circles?* Islets, 2011. **3**(6): p. 302-19.

40. MacDonald, M.J., et al., *Perspective: emerging evidence for signaling roles of mitochondrial anaplerotic products in insulin secretion.* Am J Physiol Endocrinol Metab, 2005. **288**(1): p. E1-15.

41. Wiederkehr, A. and C.B. Wollheim, *Mitochondrial signals drive insulin secretion in the pancreatic beta-cell.* Mol Cell Endocrinol, 2012. **353**(1-2): p. 128-37.

42. Cheng, K., S. Andrikopoulos, and J.E. Gunton, *First phase insulin secretion and type 2 diabetes.* Curr Mol Med, 2013. **13**(1): p. 126-39.

43. Rosengren, A.H., et al., *Reduced insulin exocytosis in human pancreatic beta-cells with gene variants linked to type 2 diabetes.* Diabetes, 2012. **61**(7): p. 1726-33.

44. Del Guerra, S., et al., *Functional and molecular defects of pancreatic islets in human type 2 diabetes.* Diabetes, 2005. **54**(3): p. 727-35.

45. Khan, A.H. and J.E. Pessin, *Insulin regulation of glucose uptake: a complex interplay of intracellular signalling pathways.* Diabetologia, 2002. **45**(11): p. 1475-83.

46. Marchetti, P., et al., *The pancreatic beta cells in human type 2 diabetes.* Adv Exp Med Biol, 2012. **771**: p. 288-309.

47. Lingvay, I., et al., *Noninvasive quantification of pancreatic fat in humans.* J Clin Endocrinol Metab, 2009. **94**(10): p. 4070-6.

48. Unger, R.H., *Lipotoxic diseases.* Annu Rev Med, 2002. **53**: p. 319-36.

49. Wang, L., et al., *Plasma fatty acid composition and incidence of diabetes in middle-aged adults: the Atherosclerosis Risk in Communities (ARIC) Study.* Am J Clin Nutr, 2003. **78**(1): p. 91-8.

50. Newsholme, P., et al., *Life and death decisions of the pancreatic beta-cell: the role of fatty acids.* Clin Sci (Lond), 2007. **112**(1): p. 27-42.

51. Maedler, K., et al., *Monounsaturated fatty acids prevent the deleterious effects of palmitate and high glucose on human pancreatic beta-cell turnover and function.* Diabetes, 2003. **52**(3): p. 726-33.

52. Diakogiannaki, E., et al., *Mechanisms involved in the cytotoxic and cytoprotective actions of saturated versus monounsaturated long-chain fatty acids in pancreatic beta-cells.* J Endocrinol, 2007. **194**(2): p. 283-91.

53. Cunha, D.A., et al., *Initiation and execution of lipotoxic ER stress in pancreatic beta-cells.* J Cell Sci, 2008. **121**(Pt 14): p. 2308-18.

54. Martino, L., et al., *Palmitate activates autophagy in INS-1E beta-cells and in isolated rat and human pancreatic islets.* PLoS One, 2012. **7**(5): p. e36188.

55. El-Assaad, W., et al., *Glucolipotoxicity alters lipid partitioning and causes mitochondrial dysfunction, cholesterol, and ceramide deposition and reactive oxygen species production in INS832/13 ss-cells.* Endocrinology, 2010. **151**(7): p. 3061-73.

56. Cnop, M., et al., *Mechanisms of pancreatic beta-cell death in type 1 and type 2 diabetes: many differences, few similarities.* Diabetes, 2005. **54 Suppl 2**: p. S97-107.

57. Maechler, P. and C.B. Wollheim, *Mitochondrial function in normal and diabetic beta-cells.* Nature, 2001. **414**(6865): p. 807-12.

58. Hellwig, C.T., E. Passante, and M. Rehm, *The molecular machinery regulating apoptosis signal transduction and its implication in human physiology and pathophysiologies.* Curr Mol Med, 2011. **11**(1): p. 31-47.

59. Orrenius, S., B. Zhivotovsky, and P. Nicotera, *Regulation of cell death: the calcium-apoptosis link.* Nat Rev Mol Cell Biol, 2003. **4**(7): p. 552-65.

60. Rahier, J., et al., *Pancreatic beta-cell mass in European subjects with type 2 diabetes.* Diabetes Obes Metab, 2008. **10 Suppl 4**: p. 32-42.

61. Clark, A., et al., *Islet amyloid, increased A-cells, reduced B-cells and exocrine fibrosis: quantitative changes in the pancreas in type 2 diabetes.* Diabetes Res, 1988. **9**(4): p. 151-9.

62. Kloppel, G., et al., *Islet pathology and the pathogenesis of type 1 and type 2 diabetes mellitus revisited.* Surv Synth Pathol Res, 1985. **4**(2): p. 110-25.

63. Sakuraba, H., et al., *Reduced beta-cell mass and expression of oxidative stress-related DNA damage in the islet of Japanese Type II diabetic patients.* Diabetologia, 2002. **45**(1): p. 85-96.

64. Marchetti, P., et al., *The endoplasmic reticulum in pancreatic beta cells of type 2 diabetes patients.* Diabetologia, 2007. **50**(12): p. 2486-94.

65. Marchetti, P., et al., *Pancreatic islets from type 2 diabetic patients have functional defects and increased apoptosis that are ameliorated by metformin.* J Clin Endocrinol Metab, 2004. **89**(11): p. 5535-41.

66. *U.K. prospective diabetes study 16. Overview of 6 years' therapy of type II diabetes: a progressive disease. U.K. Prospective Diabetes Study Group.* Diabetes, 1995. **44**(11): p. 1249-58.

67. Jeffrey, K.D., et al., *Carboxypeptidase E mediates palmitate-induced beta-cell ER stress and apoptosis.* Proc Natl Acad Sci U S A, 2008. **105**(24): p. 8452-7.

68. Back, S.H. and R.J. Kaufman, *Endoplasmic reticulum stress and type 2 diabetes.* Annu Rev Biochem, 2012. **81**: p. 767-93.

69. Schuit, F.C., P.A. In't Veld, and D.G. Pipeleers, *Glucose stimulates proinsulin biosynthesis by a dose-dependent recruitment of pancreatic beta cells.* Proc Natl Acad Sci U S A, 1988. **85**(11): p. 3865-9.

70. Eizirik, D.L., A.K. Cardozo, and M. Cnop, *The role for endoplasmic reticulum stress in diabetes mellitus.* Endocr Rev, 2008. **29**(1): p. 42-61.

71. Fagone, P. and S. Jackowski, *Membrane phospholipid synthesis and endoplasmic reticulum function.* J Lipid Res, 2009. **50 Suppl**: p. S311-6.

72. Stutzmann, G.E. and M.P. Mattson, *Endoplasmic reticulum Ca(2+) handling in excitable cells in health and disease.* Pharmacol Rev, 2011. **63**(3): p. 700-27.

73. Schroder, M. and R.J. Kaufman, *ER stress and the unfolded protein response.* Mutat Res, 2005. **569**(1-2): p. 29-63.

74. Plemper, R.K., et al., *Mutant analysis links the translocon and BiP to retrograde protein transport for ER degradation.* Nature, 1997. **388**(6645): p. 891-5.

75. Gething, M.J. and J. Sambrook, *Protein folding in the cell.* Nature, 1992. **355**(6355): p. 33-45.

76. Wang, S. and R.J. Kaufman, *The impact of the unfolded protein response on human disease.* J Cell Biol, 2012. **197**(7): p. 857-67.

77. Boyce, M. and J. Yuan, *Cellular response to endoplasmic reticulum stress: a matter of life or death.* Cell Death Differ, 2006. **13**(3): p. 363-73.

78. Oyadomari, S. and M. Mori, *Roles of CHOP/GADD153 in endoplasmic reticulum stress.* Cell Death Differ, 2004. **11**(4): p. 381-9.

79. Laybutt, D.R., et al., *Endoplasmic reticulum stress contributes to beta cell apoptosis in type 2 diabetes.* Diabetologia, 2007. **50**(4): p. 752-63.

80. Wu, J. and R.J. Kaufman, *From acute ER stress to physiological roles of the Unfolded Protein Response.* Cell Death Differ, 2006. **13**(3): p. 374-84.

81. McCullough, K.D., et al., *Gadd153 sensitizes cells to endoplasmic reticulum stress by down-regulating Bcl2 and perturbing the cellular redox state.* Mol Cell Biol, 2001. **21**(4): p. 1249-59.

82. Zong, W.X., et al., *Bax and Bak can localize to the endoplasmic reticulum to initiate apoptosis.* J Cell Biol, 2003. **162**(1): p. 59-69.

83. Scorrano, L., et al., *BAX and BAK regulation of endoplasmic reticulum Ca2+: a control point for apoptosis.* Science, 2003. **300**(5616): p. 135-9.

84. Yamamoto, K., H. Ichijo, and S.J. Korsmeyer, *BCL-2 is phosphorylated and inactivated by an ASK1/Jun N-terminal protein kinase pathway normally activated at G(2)/M.* Mol Cell Biol, 1999. **19**(12): p. 8469-78.

85. Huang, C.J., et al., *High expression rates of human islet amyloid polypeptide induce endoplasmic reticulum stress mediated beta-cell apoptosis, a characteristic of humans with type 2 but not type 1 diabetes.* Diabetes, 2007. **56**(8): p. 2016-27.

86. Sargsyan, E., et al., *Diazoxide-induced beta-cell rest reduces endoplasmic reticulum stress in lipotoxic beta-cells.* J Endocrinol, 2008. **199**(1): p. 41-50.

87. Karaskov, E., et al., *Chronic palmitate but not oleate exposure induces endoplasmic reticulum stress, which may contribute to INS-1 pancreatic beta-cell apoptosis.* Endocrinology, 2006. **147**(7): p. 3398-407.

88. Gwiazda, K.S., et al., *Effects of palmitate on ER and cytosolic Ca2+ homeostasis in beta-cells.* Am J Physiol Endocrinol Metab, 2009. **296**(4): p. E690-701.

89. Preston, A.M., et al., *Reduced endoplasmic reticulum (ER)-to-Golgi protein trafficking contributes to ER stress in lipotoxic mouse beta cells by promoting protein overload.* Diabetologia, 2009. **52**(11): p. 2369-73.

90. Romisch, K., *Endoplasmic reticulum-associated degradation.* Annu Rev Cell Dev Biol, 2005. **21**: p. 435-56.

91. Meusser, B., et al., *ERAD: the long road to destruction.* Nat Cell Biol, 2005. **7**(8): p. 766-72.

92. Kitiphongspattana, K., et al., *Proteasome inhibition alters glucose-stimulated (pro)insulin secretion and turnover in pancreatic {beta}-cells.* J Biol Chem, 2005. **280**(16): p. 15727-34.

93. Lopez-Avalos, M.D., et al., *Evidence for a role of the ubiquitin-proteasome pathway in pancreatic islets.* Diabetes, 2006. **55**(5): p. 1223-31.

94. Kawaguchi, M., et al., *Essential role of ubiquitin-proteasome system in normal regulation of insulin secretion.* J Biol Chem, 2006. **281**(19): p. 13015-20.

95. Costes, S., et al., *beta-cell dysfunctional ERAD/ubiquitin/proteasome system in type 2 diabetes mediated by islet amyloid polypeptide-induced UCH-L1 deficiency.* Diabetes, 2011. **60**(1): p. 227-38.

96. Bugliani, M., et al., *Microarray analysis of isolated human islet transcriptome in type 2 diabetes and the role of the ubiquitin-proteasome system in pancreatic beta cell dysfunction.* Mol Cell Endocrinol, 2013. **367**(1-2): p. 1-10.

97. Yorimitsu, T. and D.J. Klionsky, *Eating the endoplasmic reticulum: quality control by autophagy.* Trends Cell Biol, 2007. **17**(6): p. 279-85.

98. Masini, M., et al., *Autophagy in human type 2 diabetes pancreatic beta cells.* Diabetologia, 2009. **52**(6): p. 1083-6.

99. Las, G., et al., *Fatty acids suppress autophagic turnover in beta-cells.* J Biol Chem, 2011. **286**(49): p. 42534-44.

100. Komiya, K., et al., *Free fatty acids stimulate autophagy in pancreatic beta-cells via JNK pathway.* Biochem Biophys Res Commun. **401**(4): p. 561-7.

101. Choi, S.E., et al., *Protective role of autophagy in palmitate-induced INS-1 beta-cell death.* Endocrinology, 2009. **150**(1): p. 126-34.

102. Chen, Y.Y., et al., *Palmitate induces autophagy in pancreatic beta-cells via endoplasmic reticulum stress and its downstream JNK pathway.* Int J Mol Med, 2013. **32**(6): p. 1401-6.

103. Ebato, C., et al., *Autophagy is important in islet homeostasis and compensatory increase of beta cell mass in response to high-fat diet.* Cell Metab, 2008. **8**(4): p. 325-32.

104. Jung, H.S., et al., *Loss of autophagy diminishes pancreatic beta cell mass and function with resultant hyperglycemia.* Cell Metab, 2008. **8**(4): p. 318-24.

105. Denton, D., T. Xu, and S. Kumar, *Autophagy as a pro-death pathway.* Immunol Cell Biol, 2015. **93**(1): p. 35-42.

106. Shin, J.A., et al., *Metabolic syndrome as a predictor of type 2 diabetes, and its clinical interpretations and usefulness.* J Diabetes Investig, 2013. **4**(4): p. 334-43.

107. Reynolds, R.M., et al., *Elevated plasma cortisol in glucose-intolerant men: differences in responses to glucose and habituation to venepuncture.* J Clin Endocrinol Metab, 2001. **86**(3): p. 1149-53.

108. Bertagna, X., et al., *Cushing's disease.* Best Pract Res Clin Endocrinol Metab, 2009. **23**(5): p. 607-23.

109. Connell, J.M., et al., *Effects of ACTH and cortisol administration on blood pressure, electrolyte metabolism, atrial natriuretic peptide and renal function in normal man.* J Hypertens, 1987. **5**(4): p. 425-33.

110. Krikorian, A. and M. Khan, *Is metabolic syndrome a mild form of Cushing's syndrome?* Rev Endocr Metab Disord, 2010. **11**(2): p. 141-5.

111. Sen, Y., et al., *Children and adolescents with obesity and the metabolic syndrome have high circulating cortisol levels.* Neuro Endocrinol Lett, 2008. **29**(1): p. 141-5.

112. Stalder, T., et al., *Cortisol in hair and the metabolic syndrome.* J Clin Endocrinol Metab, 2013. **98**(6): p. 2573-80.

113. Anagnostis, P., et al., *Clinical review: The pathogenetic role of cortisol in the metabolic syndrome: a hypothesis.* J Clin Endocrinol Metab, 2009. **94**(8): p. 2692-701.

114. Karatsoreos, I.N., et al., *Endocrine and physiological changes in response to chronic corticosterone: a potential model of the metabolic syndrome in mouse.* Endocrinology, 2010. **151**(5): p. 2117-27.

115. Schacke, H., W.D. Docke, and K. Asadullah, *Mechanisms involved in the side effects of glucocorticoids.* Pharmacol Ther, 2002. **96**(1): p. 23-43.

116. Rose, A.J., A. Vegiopoulos, and S. Herzig, *Role of glucocorticoids and the glucocorticoid receptor in metabolism: insights from genetic manipulations.* J Steroid Biochem Mol Biol, 2010. **122**(1-3): p. 10-20.

117. Vegiopoulos, A. and S. Herzig, *Glucocorticoids, metabolism and metabolic diseases.* Mol Cell Endocrinol, 2007. **275**(1-2): p. 43-61.

118. Delaunay, F., et al., *Pancreatic beta cells are important targets for the diabetogenic effects of glucocorticoids.* J Clin Invest, 1997. **100**(8): p. 2094-8.

119. Newell-Price, J., et al., *Cushing's syndrome.* Lancet, 2006. **367**(9522): p. 1605-17.

120. McDonough, A.K., J.R. Curtis, and K.G. Saag, *The epidemiology of glucocorticoid-associated adverse events.* Curr Opin Rheumatol, 2008. **20**(2): p. 131-7.

121. Suissa, S., A. Kezouh, and P. Ernst, *Inhaled corticosteroids and the risks of diabetes onset and progression.* Am J Med, 2010. **123**(11): p. 1001-6.

122. Avram, D., et al., *IGF-1 protects against dexamethasone-induced cell death in insulin secreting INS-1 cells independent of AKT/PKB phosphorylation.* Cell Physiol Biochem, 2008. **21**(5-6): p. 455-62.

123. Ranta, F., et al., *Dexamethasone induces cell death in insulin-secreting cells, an effect reversed by exendin-4.* Diabetes, 2006. **55**(5): p. 1380-90.

124. Reich, E., et al., *Involvement of thioredoxin-interacting protein (TXNIP) in glucocorticoid-mediated beta cell death.* Diabetologia, 2012. **55**(4): p. 1048-57.

125. Linssen, M.M., et al., *Prednisolone-induced beta cell dysfunction is associated with impaired endoplasmic reticulum homeostasis in INS-1E cells.* Cell Signal, 2011. **23**(11): p. 1708-15.

126. Crick, F., *Central dogma of molecular biology.* Nature, 1970. **227**(5258): p. 561-3.

127. Kutlu, B., et al., *Detailed transcriptome atlas of the pancreatic beta cell.* BMC Med Genomics, 2009. **2**: p. 3.

128. Marsit, C.J., *Influence of environmental exposure on human epigenetic regulation.* J Exp Biol, 2015. **218**(Pt 1): p. 71-79.

129. Ohtsubo, K., et al., *Dietary and genetic control of glucose transporter 2 glycosylation promotes insulin secretion in suppressing diabetes.* Cell, 2005. **123**(7): p. 1307-21.

130. de Sousa Abreu, R., et al., *Global signatures of protein and mRNA expression levels.* Mol Biosyst, 2009. **5**(12): p. 1512-26.

131. Gry, M., et al., *Correlations between RNA and protein expression profiles in 23 human cell lines.* BMC Genomics, 2009. **10**: p. 365.

132. Wilkins, M.R., et al., *From proteins to proteomes: large scale protein identification by two-dimensional electrophoresis and amino acid analysis.* Biotechnology (N Y), 1996. **14**(1): p. 61-5.

133. Clamp, M., et al., *Distinguishing protein-coding and noncoding genes in the human genome.* Proc Natl Acad Sci U S A, 2007. **104**(49): p. 19428-33.

134. Lander, E.S., et al., *Initial sequencing and analysis of the human genome.* Nature, 2001. **409**(6822): p. 860-921.

135. Ahmed, M. and P. Bergsten, *Glucose-induced changes of multiple mouse islet proteins analysed by two-dimensional gel electrophoresis and mass spectrometry.* Diabetologia, 2005. **48**(3): p. 477-85.

136. Ahmed, M., J. Forsberg, and P. Bergsten, *Protein profiling of human pancreatic islets by two-dimensional gel electrophoresis and mass spectrometry.* J Proteome Res, 2005. **4**(3): p. 931-40.

137. D'Hertog, W., et al., *Two-dimensional gel proteome reference map of INS-1E cells.* Proteomics, 2011. **11**(7): p. 1365-9.

138. Waanders, L.F., et al., *Quantitative proteomic analysis of single pancreatic islets.* Proc Natl Acad Sci U S A, 2009. **106**(45): p. 18902-7.

139. Danielsson, A., et al., *The human pancreas proteome defined by transcriptomics and antibody-based profiling.* PLoS One, 2014. **9**(12): p. e115421.

140. Han, D., et al., *Detection of differential proteomes associated with the development of type 2 diabetes in the Zucker rat model using the iTRAQ technique.* J Proteome Res, 2011. **10**(2): p. 564-77.

141. Nyblom, H.K., et al., *Apoptotic, regenerative, and immune-related signaling in human islets from type 2 diabetes individuals.* J Proteome Res, 2009. **8**(12): p. 5650-6.

142. Maris, M., et al., *High glucose induces dysfunction in insulin secretory cells by different pathways: a proteomic approach.* J Proteome Res. **9**(12): p. 6274-87.

143. D'Hertog, W., et al., *Novel insights into the global proteome responses of insulin-producing INS-1E cells to different degrees of endoplasmic reticulum stress.* J Proteome Res, 2010. **9**(10): p. 5142-52.

144. Hovsepyan, M., E. Sargsyan, and P. Bergsten, *Palmitate-induced changes in protein expression of insulin secreting INS-1E cells.* J Proteomics, 2010. **73**(6): p. 1148-55.

145. Sol, E.R., M. Hovsepyan, and P. Bergsten, *Proteins altered by elevated levels of palmitate or glucose implicated in impaired glucose-stimulated insulin secretion.* Proteome Sci, 2009. **7**: p. 24.

146. Maris, M., et al., *Role of the saturated nonesterified fatty acid palmitate in beta cell dysfunction.* J Proteome Res, 2013. **12**(1): p. 347-62.

147. Merglen, A., et al., *Glucose sensitivity and metabolism-secretion coupling studied during two-year continuous culture in INS-1E insulinoma cells.* Endocrinology, 2004. **145**(2): p. 667-78.

148. Miyazaki, J., et al., *Establishment of a pancreatic β cell line that retains glucose-inducible insulin secretion: special reference to expression of glucose transporter isoforms.* Endocrinology, 1990. **127**(1): p. 126-32.

149. Ashburner, M., et al., *Gene ontology: tool for the unification of biology. The Gene Ontology Consortium.* Nat Genet, 2000. **25**(1): p. 25-9.

150. Eden, E., et al., *GOrilla: a tool for discovery and visualization of enriched GO terms in ranked gene lists.* BMC Bioinformatics, 2009. **10**: p. 48.

151. Livak, K.J. and T.D. Schmittgen, *Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method.* Methods, 2001. **25**(4): p. 402-8.

152. Wu, J., et al., *ATF6alpha optimizes long-term endoplasmic reticulum function to protect cells from chronic stress.* Dev Cell, 2007. **13**(3): p. 351-64.

153. Yamamoto, K., et al., *Transcriptional induction of mammalian ER quality control proteins is mediated by single or combined action of ATF6alpha and XBP1.* Dev Cell, 2007. **13**(3): p. 365-76.

154. Yusta, B., et al., *GLP-1 receptor activation improves beta cell function and survival following induction of endoplasmic reticulum stress.* Cell Metab, 2006. **4**(5): p. 391-406.

155. Prostko, C.R., M.A. Brostrom, and C.O. Brostrom, *Reversible phosphorylation of eukaryotic initiation factor 2 alpha in response to endoplasmic reticular signaling.* Mol Cell Biochem, 1993. **127-128**: p. 255-65.

156. Fujimoto, K., et al., *Autophagy regulates pancreatic beta cell death in response to Pdx1 deficiency and nutrient deprivation.* J Biol Chem, 2009. **284**(40): p. 27664-73.

157. Lai, E., et al., *Differential activation of ER stress and apoptosis in response to chronically elevated free fatty acids in pancreatic beta-cells.* Am J Physiol Endocrinol Metab, 2008. **294**(3): p. E540-50.

158. *Prevalence of overweight and obesity among adults with diagnosed diabetes--United States, 1988-1994 and 1999-2002.* MMWR Morb Mortal Wkly Rep, 2004. **53**(45): p. 1066-8.

159. El-Assaad, W., et al., *Saturated fatty acids synergize with elevated glucose to cause pancreatic beta-cell death.* Endocrinology, 2003. **144**(9): p. 4154-63.

160. Ortsater, H. and A. Sjoholm, *A busy cell--endoplasmic reticulum stress in the pancreatic beta-cell.* Mol Cell Endocrinol, 2007. **277**(1-2): p. 1-5.

161. Branca, R.M., et al., *HiRIEF LC-MS enables deep proteome coverage and unbiased proteogenomics.* 2013.

162. Maxfield, F.R. and I. Tabas, *Role of cholesterol and lipid organization in disease.* Nature, 2005. **438**(7068): p. 612-21.

163. Kitamura, T., *The role of FOXO1 in beta-cell failure and type 2 diabetes mellitus.* Nat Rev Endocrinol, 2013. **9**(10): p. 615-23.

164. Fu, Z., et al., *Plk1-dependent phosphorylation of FoxM1 regulates a transcriptional programme required for mitotic progression.* Nat Cell Biol, 2008. **10**(9): p. 1076-82.

165. Wang, H., et al., *The transcription factor SREBP-1c is instrumental in the development of beta-cell dysfunction.* J Biol Chem, 2003. **278**(19): p. 16622-9.

166. Ye, J., et al., *ER stress induces cleavage of membrane-bound ATF6 by the same proteases that process SREBPs.* Mol Cell, 2000. **6**(6): p. 1355-64.

167. Rutkowski, D.T., et al., *UPR pathways combine to prevent hepatic steatosis caused by ER stress-mediated suppression of transcriptional master regulators.* Dev Cell, 2008. **15**(6): p. 829-40.

168. Swagell, C.D., D.C. Henly, and C.P. Morris, *Regulation of human hepatocyte gene expression by fatty acids.* Biochem Biophys Res Commun, 2007. **362**(2): p. 374-80.

169. Kakuma, T., et al., *Leptin, troglitazone, and the expression of sterol regulatory element binding proteins in liver and pancreatic islets.* Proc Natl Acad Sci U S A, 2000. **97**(15): p. 8536-41.

170. Silverstein, A.M., et al., *Protein phosphatase 5 is a major component of glucocorticoid receptor.hsp90 complexes with properties of an FK506-binding immunophilin.* J Biol Chem, 1997. **272**(26): p. 16224-30.

171. Zuo, Z., et al., *Ser/Thr protein phosphatase type 5 (PP5) is a negative regulator of glucocorticoid receptor-mediated growth arrest.* Biochemistry, 1999. **38**(28): p. 8849-57.

172. Chan, J.Y., et al., *Failure of the adaptive unfolded protein response in islets of obese mice is linked with abnormalities in beta-cell gene expression and progression to diabetes.* Diabetes, 2013. **62**(5): p. 1557-68.

173. Ichijo, H., et al., *Induction of apoptosis by ASK1, a mammalian MAPKKK that activates SAPK/JNK and p38 signaling pathways.* Science, 1997. **275**(5296): p. 90-4.

174. Miller, A.L., et al., *p38 Mitogen-activated protein kinase (MAPK) is a key mediator in glucocorticoid-induced apoptosis of lymphoid cells: correlation between p38 MAPK activation and site-specific phosphorylation of the human glucocorticoid receptor at serine 211.* Mol Endocrinol, 2005. **19**(6): p. 1569-83.

175. Rogatsky, I., S.K. Logan, and M.J. Garabedian, *Antagonism of glucocorticoid receptor transcriptional activation by the c-Jun N-terminal kinase.* Proc Natl Acad Sci U S A, 1998. **95**(5): p. 2050-5.

176. Bouazza, B., et al., *Cytokines alter glucocorticoid receptor phosphorylation in airway cells: role of phosphatases.* Am J Respir Cell Mol Biol, 2012. **47**(4): p. 464-73.