# Mechanisms responsible for cytokine and hyperglycemia induced β-cell dysfunction

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# ABSTRACT

*Aims/hypotheses*: The two main forms of diabetes, type 1 and type 2, are characterised by progressive β-cell failure. β-cell failure in both types of diabetes is characterised by functional defects [selective loss of glucose stimulated insulin secretion (GSIS)], and reduced β-cell mass due to increased cell death (apoptosis). Hyperglycaemia and increased cytokines are likely causes of the loss of GSIS and β-cell apoptosis but the mechanisms responsible remain unknown. This Master thesis examined the two separate hypotheses: *firstly*, that hyperglycaemia leads to endoplasmic reticulum (ER) stress in pancreatic β-cells and this contributes to increased apoptosis; and *secondly*, that cytokines lead to β-cell dedifferentiation and this contributes to the loss of GSIS.

*Methods*: Studies were performed in MIN6 β-cells and in isolated islets from two different mouse strains, C57BL/6J and DBA/2. Islets were handpicked after pancreas digestion. Islets and MIN6 cells were treated with different concentrations of glucose over a time course ranging from 4 to 72 h. At the end of the treatment period, either apoptosis was measured or RNA was extracted and mRNA levels of candidate ER stress genes assessed by real-time PCR. MIN6 cells were treated with cytokines (either IL-1β alone or co-treatment with IL-1β, IFN-γ and TNF-α) for 24 or 48 h, and either insulin secretory function was evaluated or RNA was extracted and mRNA levels of candidate β-cell differentiation genes assessed by real-time PCR.

*Results*: Surprisingly, only a modest increase in apoptosis was observed in MIN6 cells cultured at high glucose. By far the largest increase in apoptosis was observed in MIN6 cells cultured in low glucose medium. In both isolated islets and MIN6 cells, high glucose treatment induced ER stress, as evidenced by upregulation of several genes specific to the unfolded protein response (BiP, ERP72, EDEM1, P58) and increased processing of XBP-1, a transcription factor which is entirely dependent on activation of UPR transducer protein IRE1 as a consequence of ER stress. Upregulation of these ER chaperones, folding enzymes and degradation proteins (BiP, ERP72, EDEM1) would serve to protect the cells from further endoplasmic reticulum stress and apoptosis. On the other hand, MIN6 cells and islets treated with low glucose levels displayed increased mRNA levels of the apoptosis inducer CHOP, which appeared to be independent of ER stress and likely mediated by the integrated stress response.

Chronic treatment of MIN6 cells with cytokines led to a reduction in GSIS. This was associated with reduced mRNA levels of several islet associated transcription factors (Pax6, HNF4α, PDX-1,

Nkx6.1, BETA2). Moreover this was also associated with alterations in mRNA levels of many genes implicated in β-cell glucose sensing (GLUT2, mGPDH, Kir6.2, SERCA2b). Conversely, several genes that are normally suppressed in β-cells such as Id-1 and iNOS that would theoretically impair β-cell function were increased. The severities of the changes in β-cell gene expression, apoptosis, and insulin secretion were dependent on the time of exposure to hyperglycaemia and cytokines.

*Conclusions/interpretation*: These studies demonstrate that hyperglycaemia induces ER stress in β-cells with UPR activation providing protection from apoptosis. Conversely, hypoglycaemia induces apoptosis which is associated with increased CHOP. Thus, ER stress plays a critical role in the survival of β-cells exposed to abnormal glucose levels.

Cytokines lead to alterations in the pattern of islet gene expression consistent with the hypothesis that a gradual loss of differentiation contributes to the dysfunction of β-cells in diabetes. Noteworthy, it needs further performance to confirm if these results are statistically significant.

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# ABBREVIATIONS

ATF4 - activating transcription factor 4

ATF6 - activating transcription factor 6

ATP - adenosine triphosphate

BETA2 - β-cell E-box trans-activator 2

bHLH - basic helix loop helix

BiP - immunoglobulin heavy chain binding protein

Cdk - cyclin-dependent kinase

CHOP - C/EBP homologous protein

CPT-1 - carnitine palm itoyl transferase 1

DMEM - Dulbeccos modified Eagles medium

DM - diabetes mellitus

dNTP - deoxynucleotidtriphosphate

elF2α - eukaryotic translation initiation factor 2α

EDEM - endoplasmic reticulum degradation enchanching α-mannodiase-like protein

ER - endoplasmic reticulum

ERAD - Endoplasmic reticulum associated degradation

ERP72- endoplasmic reticulum protein 72

FACS - Fluorescence Activated Cell Sorting

fa - fatty acid

GSIS - glucose stimulated insulin secretion

GK - glucokinase

GLUT2 - glucose transporter-type 2

GRP94 - glucose-regulated protein 94

HNF - hepatocyte nuclear factor

IAPP - islet amyloid polypeptide

ID-1 - inhibitor of differentiation/DNA binding

IGF - insulin-like growth factor

INFγ - intermediate nuclear factor γ

INOS - inducible nitric oxide synthase

IRE1 - inositol requiring enzyme 1

IRS - insulin receptor substrate

JNK - C-jun N-terminal kinase

Kir6.2 - pore-forming subunit of the ATP-sensitive $K^+$ channel

KRB - Krebs-Ringer bicarbonate

LC-CoA - long-chain acetyl-CoA

LDH-A - lactate dehydrogenase

mGPDH - mitochondrial glycerol-3-phophate dehydrogenase

MODY - Maturity onset diabetes of the young

mRNA - messenger ribonucleotide acid

NADH - nicotinamide adenine dinucleotide

NF-kappaB - nuclear factor kappaB

Nkx6.1 - NK6 transcription factor related, locus 1

ob - obese

P58 - 58 kDa inhibitor of double stranded activated protein kinase PKR

Pax6 - paired box gene 6

PC - pyruvate carboxylase

PCR - polymer chain reaction

PDX-1 - pancreatic and duodenal homeobox-1

PERK - PKR-like endoplamic reticulum kinase

PKC - Protein kinase C

Px - pancreatectomized rats

RIA - radioimmunoassay

ROS - reactive oxygen species

RT - reverse transcriptase

RT-PCR - Real-Time Polymer Chain Reaction

SERCA2b - sarco endoplasmic reticulum $Ca^{2+}$ transport ATPase 2b

SUR1 - sulfonylurea receptor 1

STAT - signal transducer and activator of transcription

TCA cycle - tricarboxylic acid cycle

UCP-2 - uncoupling protein-2

UPR - unfolded protein response

VDCC - voltage-dependent $Ca^{2+}$ channel

Xbp-1 - X-box binding protein 1

ZDF rat - Zucker diabetic fatty rat

# 1 INTRODUCTION

Diabetes Mellitus (DM) is the most common metabolic disorder. The past two decades have seen an explosive increase in the number of people diagnosed with diabetes worldwide; estimated from 151 millions in 2001 to 221 millions in 2010 (Zimmet, Alberti et al. 2001). DM is one of the diseases that cost the community most because of high mortality as well as medical complications. DM leads to many late dangerous complications such as obesity, kidney failure, eyes nefrophati, diabetes foot, macro and micro vascular disease.

There are two main types of Diabetes Mellitus. Both types are characterized by progressive β-cell failure. Type 1diabetes (T1D) or insulin dependent DM is due primarily to autoimmune mediated destruction of pancreatic β-cells islet, resulting in absolute deficiency. The pathogenesis of T1D is not fully clear, but many studies have suggested that the environment eg. viral infection, toxins of dietary nitrate and nitrite, streptomyces toxin, early infants diet of cows milk and vaccine administration, may trigger activation of the autoimmune mechanisms in genetically susceptible individuals (Zimmet, Alberti et al. 2001).
Type 2 diabetes (T2D) is characterized by insulin resistance and/or abnormal insulin secretion. Factors like sex, age, physical activity, diet and stress may contribute to the development of the disease.
T1D is the most common chronic disease of children and T2D is primarily confined to adult, but is increasingly prevalent in children and adolescents lately, due to heightened genetic susceptibility of certain ethnic groups (Zimmet, Alberti et al. 2001).

## 1.1 Development of Diabetes mellitus

Insulin resistance at the level of peripheral tissues and liver associated with obesity or in the early stages of T2D, a compensatory response can involve both an increase in islet β-cells volume (hypertrophy and hyperplasia) (Butler, Janson et al. 2003) and a lowering of the threshold for glucose stimulated insulin secretion (GSIS) (Weir, Bonner-Weir et al. 2004). It is the failure of β-cell compensation that is fundamental to the development of T2D. β-cell failure is characterised by defective secretory function and morphological alterations. The primary secretory defect is a selective loss of GSIS which has been identified in early stage of T1D, T2D, many animal models and also in recipient of islet transplants (Gray, Cranston et al. 1998; Ogawa, Noma et al. 1995; Leahy, Bonner-weir et al. 1992). Despite its fundamental importance to diabetes pathology, the

underlying mechanism(s) for the loss of GSIS remains unknown. Beside from secretory dysfunction, β-cell mass is reduced in people with diabetes compared to weight-matched subjects without diabetes (Butler, Janson et al. 2003). This deficiency in β-cell mass has been linked to increased rate of β-cell apoptosis (Butler, Janson. et al. 2003; Pick, Clark et al. 1998; Shimabukuro, Zhou et al. 1998). In the study by Butler, Janson et al. 2003, pancreatic tissue from autopsies was examined and the results shows that β-cell volume was significantly increased in obese versus lean subjects. There was no difference of β-cell replication among groups, but the neogenesis was increased in obesity group compared to other groups. However, the frequency of β-cell apoptosis was increased 10-fold in lean and 3-fold in obese case of T2D compared with their respective nondiabetic control group.

It is believed that β-cells dysfunction and death occurs as the combined consequence of increased circulating glucose and saturated fatty acid together with adepocyte secreted factors and activation of the innate immune system.

When β-cells fail to adequately compensate for increased demand for insulin and the plasma glucose level starts to increase above the normal range (> 7 mM), then the diabetic stage is evident. But only 20 % of those people with severe insulin resistance become diabetic, with the other 80 % being able to maintain near normal glycemic level (Bonner-Weir S. 2000).

## 1.2 BETA-CELL FUNCTION AND PHYSIOLOGY

### 1.2.1 β-cell dedifferentiation in diabetes

The β-cell is a highly specialised cell. In addition to gene products involved in the synthesis, processing and secretion of insulin, it possesses a unique metabolic profile allowing it to respond to nutrient secretogogues over their physiological concentration range (Halban, Kahn et al. 2001; Deneey, Prentki et al. 2000). This highly differentiated state is maintained by an as yet poorly understood hierarchy of transcription factors (Servitja and Ferrer 2004; Huang, Tsai et al. 2000). Of the major intracellular steps leading to GSIS no single defect has been identified that can explain, in full, the impairment in β-cell function. In this proposal, we hypothesise that inadequate β-cell insulin secretion is caused by a loss of the unique expression pattern of genes responsible for maintaining GSIS. Studies have found increases for a gene capable of inhibiting differentiation, inhibitor of dedidiferentiation/DNA binding (Id-1), and for genes implicated in endoplasmic reticulum (ER) stress, a novel potential mechanism for β-cell destruction in type 1 and type

2 diabetes. From these studies in diverse animal models we therefore propose that: with exposure to hyperglycaemia, β-cells develop a phenotypic alteration that, initially results in dedifferentiation and the loss of GSIS, but with sustained insult ultimately leads to apoptosis.

### 1.2.2 Cellular structure of the β-cells in the pancreatic islet

β-cells are an integral part of islet of Langerhans in pancreas. A normal adult human pancreas contains about 1 million individual islets which amount to 1-2 % of the organ mass (Newgard and Matschinsky 2000). The islets contain four types of endocrines cells, an intricate array of nerves terminals, and a very dense mesh of microvasculature. The four types of endocrine cells are the β-cell (70 % of all cells), which secretes insulin and islet amyloid polypeptide, the α-cell that secretes glucagon, the γ-cell that secretes somastatin, and the PP-cell that secretes pancreatic polypeptide (Bishop and Polak 1997).

Insulin is an inhibitor of glucagon secretion, while glucagon stimulates insulin secretion, and somastatin inhibits both insulin and glucagon secretion. It has been suggested that blood flow to the islet via arterioles of the microvasculature arrives at the β-cell core first, and then ramifies to the periphery of the islets where the other cells types reside. When insulin secretion is stimulated, insulin-laden blood flowing past α-cells would serve to inhibit glucagon secretion, such an arrangement would help to ensure maximal metabolic efficiency of the secreted insulin (Newgard and Matschinsky 2000).

## 1.3 INSULIN BIOSYNTHESIS AND SECRETION

### 1.3.1 The effect of insulin

Insulin is a protein with a covalently connected polypeptide chains produced as a prehormon in the rough endoplasmic reticulum and is the main hormone controlling glucose metabolism. Insulin is secreted by the pancreatic β-cells, and it affects every tissue in the body but principally the liver, muscle and adipocytes. Insulins overall effect is the conversation of the body fuel supplies. It increases the rate of glycogen synthesis and triglyceride formation, due to increased uptake of glucose and fatty acids. Insulin also inhibits the catabolic breakdown of already existing energy stores of glycogen and fat. Additionally, insulin increases protein synthesis in liver, muscles and adipocytes as a part of its activation of anabolic pathways (Nelson and Micheal 2000).

### 1.3.2 Glucose stimulated insulin secretion (GSIS)

The β-cells respond to both the actual glucose concentration and also to the rate of change of blood sugar. There are three possible pathways by which glucose can stimulate insulin secretion.

- One is the $K^+$ ATP-dependent pathway where the metabolic breakdown of glucose via glycolysis and the TCA cycle is essential (discussed under).
This pathway is the most studied and accepted mechanism for triggering GSIS.
- The second pathway is the anaplerotic/malonyl-CoA pathway. This theory suggests that glucose leads to a rise in malonyl CoA, which inhibits carnitine palmitoyltransferase (CPT-1) thereby preventing mitochondrial fatty acid uptake and oxidation.
It has been proposed that the resultant accumulation of long-chain acyl-CoA (LCCoA) in the cytosol, can activate insulin secretion in a variety of ways including stimulation of protein kinase C (Komatsu, Yajima et al. 1999).
- The third pathway is the $K^+$ ATP-independent pathway, where a number of signals, including glutamate have been identified as factors promoting exocytosis of insulin secretory granules and insulin release (Maechler and Wollheim 1999).
Generally, the anaplerotic and the $K^+$ ATP-independent pathways are less well defined, and are not emphasised here as important for acute insulin secretion in comparison to the $K^+$ ATP-dependent pathway.

The $K^+$ ATP-dependent pathway (figure 1):
Glucose stimulates insulin secretion by entry into β-cells achieved by $Na^+$-independent facilitated glucose transporter, which is a family of GLUTs 1-5 gene family. Where GLUT2 has the highest affinity for glucose.
Inside the β-cell glucose is phosphorylated to glucose-6-P by glucokinase which is a member of the hexokinases gene family. ATP is the important coupling factor that activates further ion-channels for insulin secretion, and it can be made in three different pathways, produced by glucose metabolism. 1) Through NADH produced in the glyceraldehydes phosphate dehydrogenase, reaction can be transferred to the mitochondria for entry into the electron transport chain via α-glycero-P and aspartate/malate shuttles. 2) Through phosphoglycerate kinase and pyruvate kinase reactions of glycolysis. 3) Via mitochondrial oxidation of pyruvate (Newgard and Matschinsky 2000).

Rise in ATP stimulates insulin secretion by conductance of adenine nucleotide-sensitive $K^+$ channels which depolarized the β-cell membrane and open the voltage sensitive $Ca^{2+}$ channel into the β-cell. The influx of $Ca^{2+}$ activates insulin secretion (Newgard and Matschinsky 2000).

Insulin secretion is primarily regulated by glucose concentration although other signaling substance are involved, including amino acids, fatty acids, hormones growth factors, certain pharmakologic agents and other carbohydrates may trigger insulin secretion (Prentki M. 1996).

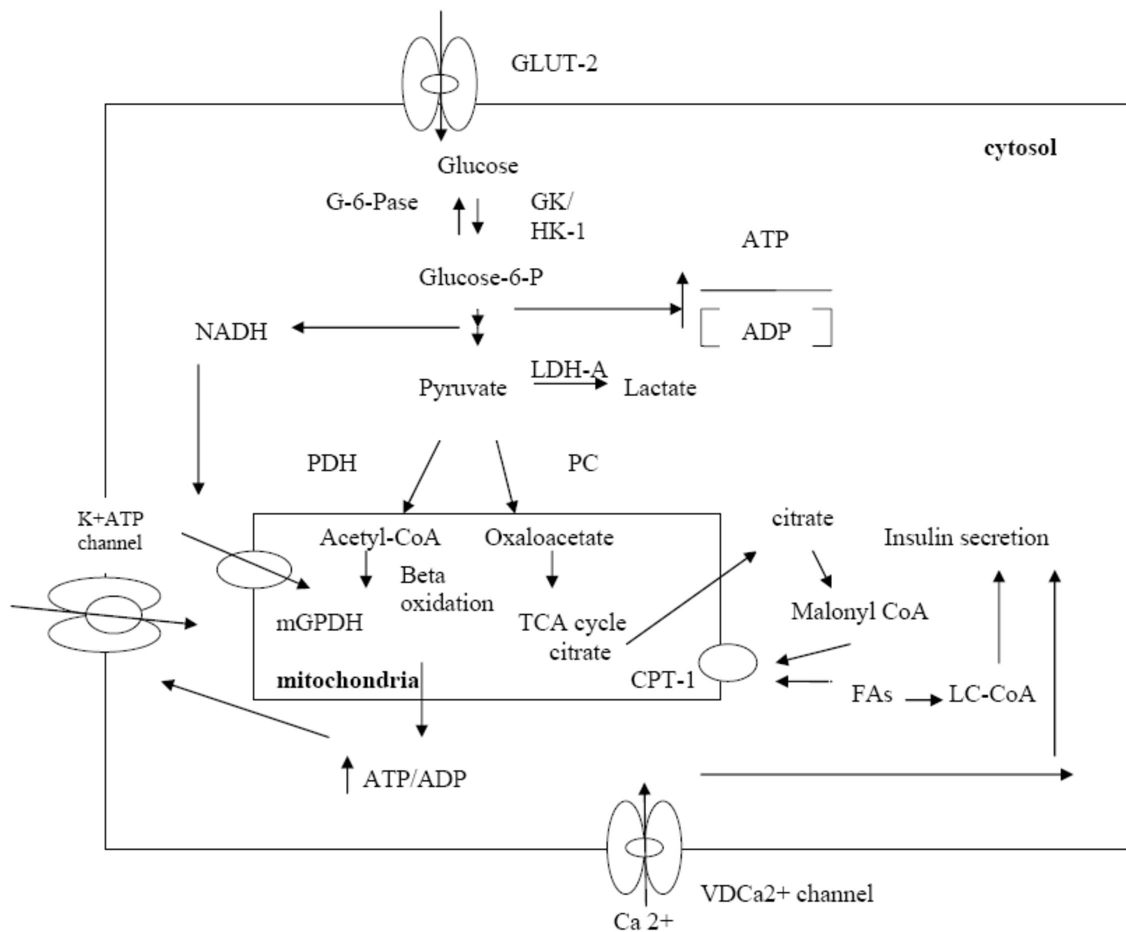


**Figure 1. Schematic representation of glucose-induced insulin secretion**

Glucose enters the β-cell via GLUT2 where it is phosphorylated by GK (glucokinase) andHK-1 (hexokinase-1). The metabolic breakdown of glucose through glycolysis leads to production of NADH, which is transported through the mGPDH (mitochondrial glycerol-3-phosphate dehydrogenase) shuttle to the mitochondrial electron transport chain. Metabolism of glucose increases ATP/ADP ratio which leads to opening of the ATP-sensitive $K^+$-channel and subsequently opening of voltage-dependent calcium channels (VDCC) which stimulate insulin secretion. The product of glycolysis, pyruvate can be carboxylated via pyruvate carboxylase (PC) or converted to lactate via lactate-dehydrogenase (LDH-A). Carboxylation of pyruvate, replenishes intermediates of the TCA cycle, leading to increased citrate which can be converted to malonyl-CoA. Malonyl-CoA has an inhibitory effect on the fatty acid transporter CPT-1 (carnitine palmitoyl transferase 1) causing accumulation of fatty acids in cytosol and acute stimulation of insulin release. (figure is taken from Kjørholt C. thesis 2003).

## 1.4 SPECIALIZATION OF β-CELL GENE EXPRESSION AND METABOLISM

### 1.4.1 Regulation of insulin transcription

In the multistep process of insulin synthesis it is the initiation of transcription that restricts insulin synthesis to the β-cell, since all subsequent steps can be performed by other cell types (Sander and German 1997). The β-cell-specific transcription of insulin is assumed to be a synergistic interaction of a unique set of regulatory proteins and a substitution or a deletion of any of these factors reduces the insulin promoter activity (Sander and German 1997). The insulin promoter region is highly conserved among species and contains a number of regulatory elements. Most focus has been on the E box and A box elements because key transcription factors bind to these regulatory elements. There are two box elements (E1 and E2) that bind transcription factor heterodimers consisting of the ubiquitous class A and a tissue-specific class B member of the basic helix loop helix (bHLH) family (see figure 2). Mutation of either of the two elements results in a 90 % loss of promoter activity and a double mutation of the two elements affectively abolishes transcription (Sander and German 1997). The E2 element binds among others the transcription factor beta-cell E-box trans-activator 2 (BETA2), which is also known as NeuroD (Naya, Stellrecht et al. 1995). The importance of BETA2/neuroD is emphasized by studies showing that mice homozygous for BETA2/neuroD gene deletion develop severe diabetes and die 3-5 days after birth (Naya, Huang et al. 1997). The homeodomain transcription factor PDX-1 (also known as IDX-1, STF-1, IPF-1), binds to the A-box elements will be discussed under.

### 1.4.2 The transcription of genes involved in GSIS and β-cell differentiation

Many studies have investigated the transcription factor regulation of the insulin gene, and there are indications that the same transcription factors play an important regulatory role in transcription of other key β-cell genes and suppress many others (see figure 3). The pancreatic duodenal homeobox gene-1 (PDX-1) is a master regulator of both pancreatic development and the differentiation of cells into the β-cell phenotype (Stoffers, Thomas et al. 1997). It was identified as a β-cell specific regulatory factor for transcriptional expression of insulin genes and has been shown to regulate the expression of islet- specific genes, including GLUT-2 (Waeber, Thompson et al. 1996) and glucokinase both genes of β-cell metabolism (Watada, Katjimoto et al. 1996). NK6 transcription factor related, locus 1 (Nkx6.1) is a transcription factor which regulates the differentiation of the pancreatic endocrine cells, and is a member of the NK2 family of homeoprotein transcription factors (Sussel, Kalamaras et al. 1998). Nkx6.1 is expressed primarily in β-cells of adult islets (Jensen, Serup et al. 1996).

Both PDX-1 and Nkx 6.1 have been found to be expressed in the pancreatic bud. Studies with mice homozygous for a targeted mutation in the PDX-1 gene, reported a lack of pancreas (Jonsson, Carlsson et al. 1994; Huang, Tsai et al. 2000) and in a human subject with a homozygous mutation in the PDX-1 gene the pancreas failed to develop (Stoffers, Zinkin et al. 1997). Accordingly, these findings have given suggestions that PDX-1 function both in the early specification of the primitive gut to a pancreatic fate and in the maturation of the pancreatic β-cell (Guz, Montminy et al. 1995). Recent study has shown that the pancreas Nkx6.1 mutant mice have reduced insulin producing cells but other islets cells are developing normally (Henseleit, Nelson et al. 2005).
The two transcription factors BETA-2/NeuroD and paired box gene 6 (Pax6) have been found to be expressed in the early developing pancreas (Sander and German 1997), but their role in islet cell differentiation is not known.
Isulin receptor substrate 2 (IRS-2), which is an insulin receptor substrate appears to be the key in maintaining optimal β-cell mass to compensate for peripheral insulin resistance (Withers, Gutierrez et al. 1998; Withers, Burks et al. 1999). IRS-2 is activated by the insulin growth factor (IGF) (Lingohr, Buettaer et al. 2002).
The importance of the transcription factors in the pathogenesis of diabetes is in focus because of the discovery of several mutations in specific transcription factors involved in subtypes of diabetes. Maturity Onset Diabetes of the Young 4 (MODY4) has a mutation in the transcription factor PDX-1 (Stoffers, Thomas et al. 1997). There have also been described mutations in the genes encoding glucokinase (MODY2), and the transcription factor BETA2/NeuoD has recently also been linked to MODY6 (Servitja and Ferrer 2005). MODY 1, 3 and 5 has been linked to mutation in the hepatocyte nuclear factor (HNF) homeodomain family (Habener, Kemp et al. 2005).

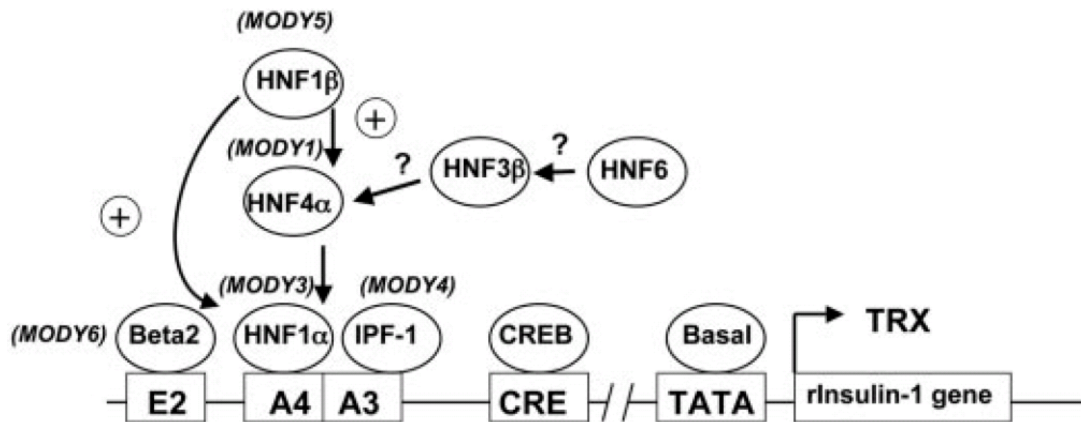


Figure 2. Transcription factor cascade involved in the regulation of the rat insulin-1 gene promoter The enhancers E2-A4/A3 (and E1/A1, not shown) bind and are activated by bHLH proteins and the homeodomain protein Ipf-1 (PDX-1). HNF1α, also binds to the E2-A4/A3 complex. Hnf4, in turn, is regulated by Hnf1α and possibly Hnf3β (activated by Hnf6). Mutations in Hnf4α, Hnf1α, Ipf-1, Hnf1α, and Beta2 are responsible for Maturity Onset Diabetes of the Young (MODY) 1, -3, -4, -5, and -6, respectively. CREB, cAMP response element-binding protein; Basal, basal transcription factors; Trx, transcriptional activation of rat insulin-1 gene promoter (rInsulin-1 gene), (figure taken from article*: transcriptional regulation in pancreatic development*, Habener, Kemp et al. 2005).

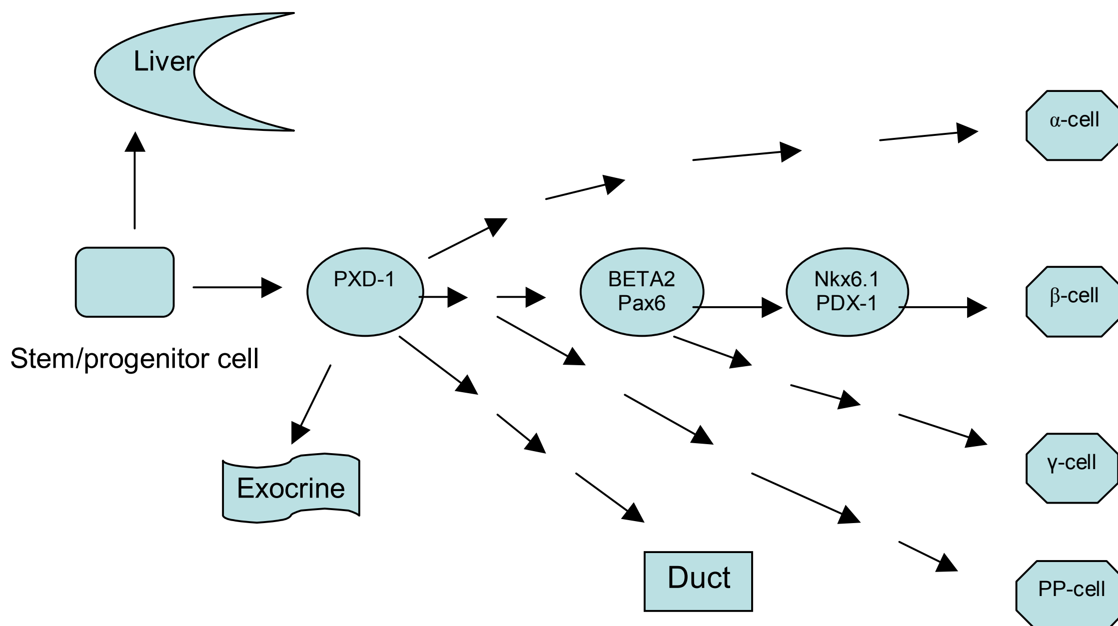


**Figure 3. Schematic diagram of the differentiation of pancreatic β-cells**

Optimal secretory function is dependent upon the differentiation of β-cells maintained by a set of transcription factors, in which some of them are identified to be important at different stages of β -cell differentiation. The pancreatic duodenal homeobox gene-1 (PDX-1), paired box gene 6 (Pax6), β-cell E-box trans-activator 2 (BETA2), NK6 transcription factor related, locus 1 (Nkx6.1).

### 1.4.3 Inhibitor of differentiation/DNA binding, Id-1

Id-1 is a member of a family of proteins associated, in other cell types, with cell growth, enhanced proliferation and dedifferentiation (Sikder, Devlin et al. 2003; Yokota and Mori 2002). Id proteins are negative regulators of helix-loop-helix (HLH) transcription factors (Benezra, Davis et al. 1990) but can also act via non-HLH proteins (Norton J.D. 2000). It is noteworthy that HLH transcription factors are critical for β-cell development and function (Huang, Tsai et al. 2000; Naya, Huang et al. 1997; Cordle, Henderson et al. 1991). Id proteins have been studied as proto-oncogenes but they are also implicated in normal cell cycle progression (Sikder, Devlin et al. 2003; Yokota, Mori et al. 2002). Recently, Laybutt and co found hyperglycaemia-induced expression of Id-1 in islets of diabetic db/db mice in association with reduced expression of β-cell transcription factors, glucose sensing genes and insulin content indicative of a loss of β-cell differentiation (Laybutt, Sharma et al. 2002). The selective lowering of hyperglycaemia with phlorizin led to a restoration of secretory function, Id-1 and β-cell differentiation gene expression, and insulin content (unpublished paper by Laybutt et al. 2005), thus further highlighting the validity of this relationship. Furthermore, in hyperglycaemic pancreatectomized (Px) rats, Laybutt and co have similarly found Id-1 expression to be associated with β-cell dedifferentiation, reduced glucose-induced ATP synthesis and a loss of GSIS (Kateno, Sharma et al. 2002; Scheuner, Song et al. 2001; Ruderman, Saha at al. 1998). Moreover, *in vitro* studies have demonstrated that glucose regulates the expression of Id-1 in human islets and insulin-secreting cell lines but not in liver or other non-β-cell lines (Webb, Akbar et al. 2001; Wice, Bernal-Mizrachi et al. 2001).

## 1.5 THE ROLE OF GLUCOSE AND CYTOKINES ON THE β-CELL FUNCTION

### 1.5.1 The role of hyperglycemia in β-cell apoptosis and dysfunction

Chronic hyperglycemia is the factor that may cause tissue dysfunction. Animal study of partially pancreatectomized rats is supporting the hypothesis that chronically exposing of normal β-cell to an elevated plasma glucose level will make that cell become non-responsive to glucose, the so called glucose toxicity and it is linked to loss of GSIS (Leahy, Bonner-Weir et al. 1992). Chronic hyperglycemia also leads to a critical loss of β-cell differentiation with altered expression of genes involved in multiple metabolic pathways diversionary to normal β-cell glucose metabolism. This global maladaptation in gene expression at the time of increase secretory demand may contribute to the β-cell dysfunction found in diabetes (Laybutt, Sharma et al. 2002). Elevated glucose (and fatty acids level and oxidative stress) are believed to leads to β-cell dysfunction and the induction of

β-cell apoptosis by triggering apoptosis factors from mitochondria (Donath, Ehses et al. 2005). It is believed that hyperglycemic activate of the immune system causes an inflammation and release of the inflammation factors or cytokines, such as interleukin (IL)-1β, tumor necrosis factor (TNF)-α and interferon (IFN)-γ witch are believed to cause β-cell dysfunction and dead (Cnop, Welsh et al. 2005). Endoplasmic reticulum (ER) stress has also recently emerged as a potential mediator (Cardozo, Ortis et al. 2005; Kharroubi, Ladriere et al. 2004).

In addition hyperglycemia exposure in β-cell over time can lead to oxidative stress and generation of reactive oxygen species (ROS) which ultimately could lead to tissue damage and cell apoptosis (Orthega-Camarillo, Guzman-Grenfell et al. 2006; Evans, Goldfine et al. 2002; Biares, Montolio et al. 2002; Marshak, Leibowitz et al. 1999). ROS can also reduce insulin secretion by pancreatic β-cells (Sakai, Matsumoto et al. 2003; Evans, Goldfine et al. 2002).

### 1.5.2 Potential mechanisms of hyperglycemia-induced β-cell dysfunction

We hypothesise that a gradual rise in glucose levels triggers two parallel pathways leading to β-cell dysfunction. One arm signals an enhanced demand for insulin via increased Id-1 expression, which normally participates in the growth/proliferation signalling in the β-cell. However, the trade off is a loss of secretory function via Id-1 induced dedifferentiation. In the second arm, we hypothesise that high glucose leads to the misfolding of ER proteins, thus inducing ER stress. β-cells are thought to be highly susceptible to this because of their heavy commitment to insulin synthesis. Initially, cells have a self-protective mechanism against ER stress involving translational attenuation as well as the activation of a specific transcriptional program that facilitates handling of the insulin secretory cargo. With severe and prolonged hyperglycaemia such as occurs in diabetes and after islet transplantation, further dedifferentiation and ER stress ensues, with the unfolded protein response (UPR) signalling switching to a cell death program leading to β-cell apoptosis.

### 1.5.3 The role of cytokines in β-cell dysfunction and apoptosis

Activation of type 1 cytokines such as interleukins 1-β (IL1-β), tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) can contribute to the development of β-cell dysfunction. This has been proven by many studies (Cordozo, Ortis et al. 2005; Kharroubi, Ladriere et al. 2004; Ling, Van de Casteele et al. 2000). The mechanisms behind it are thought to be repression of β-cell maturation and insulin secretion and increase expression of gene capable of inhibiting differentiation namely Id-1 and genes implicated in ER-stress and cell apoptosis.

Rodent studies where β-cell where treated by cytokines shows an increase expression of genes that are connected to ER-stress (Ling, Van de Casteele et al. 2000). Cytokines are believed to induce

β-cell apoptosis via the activation of β-cell genes network under the control of the transcription factors nuclear factor kappaB (NF–κB) and signal transducer and activator of transcription (STAT-1). The exact pathway of STAT-1 via intermediate nuclear factor γ (INF-γ) to apoptosis is still unclear (Cnop, Welsh et al. 2005).
It has been suggested that IL-1β induced NF–κB activation triggers production of nitric oxide (NO), chemokines and depletion of endoplasmic reticulum calcium store, possibly by reducing expression of the sarco/endoplasmic reticulum $Ca^{2+}$-ATPase pump (Serca2b) (Oyardormari, Akari et al. 2002). Prolonged depletion in ER $Ca^{2+}$ is a form of ER stress, in which an apoptosis program is activated (Cnop, Welsh et al. 2005).

### 1.5.4 ER-stress

As mentioned above one mechanism by which toxic nutreats and cytokines may induce β-cell dysfunction is via endoplasmic reticulum (ER) stress.
Pancreatic β-cells process a highly developed ER, probably reflecting heavy engagement in the folding, export and processing of newly synthesized insulin. ER stress is the protection condition against cell ER disruption. Various conditions that disrupt ER function lead to the accumulation of misfolded proteins in the ER including inhibition of protein glycosylation, reduction of formation of disulfide bonds, ER $Ca^{2+}$ depletion from the ER lumen, impairment of protein transport from the ER to the Golgi, and expression of malfolded proteins (Zhang, Kaufman et al. 2004; Oyadomari, Koizumi et al. 2002; Harding and Ron 2002).This trigger an evolutionary-conserved, adaptive program that involves four functionally distinct responses:
1. Translational attenuation to reduce the synthesis of new protein and to prevent further accumulation of unfolded proteins;
2. Up-regulation of the genes encoding ER chaperone proteins to increase protein folding activity and to prevent protein aggregation;
3. Proteosomal degradation of misfolded proteins following their regulated extrusion from the ER socalled ER associated depletion (ERAD); and
4. Apoptosis in the event of persistent stress.

The signalling pathways underlying this program, whereby information is relayed from the ER to the nucleus, are known as the unfolded protein response (UPR). Immunoglobulin heavy chain binding protein (BiP) is central to this overall process as it serves as an ER chaperone and a sensor of protein misfolding (Bertolotti, Zhang et al. 2000). In non-stressed cells, BiP associates on the ER luminal surface with three UPR transducer proteins, inositol requiring enzyme 1 (IRE1), activating transcription factor 6 (ATF6) and PKR-like endoplasmic reticulum kinase (PERK), and maintains

these proteins in inactive forms. Under stressed conditions, BiP dissociates from the transducer proteins inducing their activation and subsequent up-regulation of UPR target genes (BiP, EDEM1, ERP72, ATF4, CHOP and P58), as well as translational attenuation due to phosphorylation of the eucaryotic translation initiation factor 2α (eIF2α) by the protein kinase PERK (see figure 4). eIF2α is also a substrate for other stress-activated protein kinases so its phosphorylation is thus also a feature of the so-called integrated stress response. When functions of the ER are severely impaired, apoptosis is induced by transcriptional activation of the gene for C/EBP homologuos protein (CHOP/GADD153) (Oyadomari, Koizumi et al. 2002; Wang, Lawson et al. 1996), and by activation of the C-jun-N-terminal kinase (JNK) pathway and caspase-12. (Harding, Ron et al. 2002; Oyadomari, Araki et al. 2002).
The transcription factor X-box binding protein 1 (XBP-1), has been reported to transcribe gene specifically involved in the mammalian UPR (Yoshida, Matsui et al. 2001). XBP-1 mRNA is spliced in response to ER stress via IRE1 activation which gives a higher transcription in the up-regulation of UPR target genes (Oyadomari, Akari et al. 2002). XBP-1 is believed to be activated at high level in the late phase of ER stress (Yoshida, Masui et al. 2001).

### 1.5.5 ER stress and the β-cell

It has been demonstrated in PERK-deficient mice (Harding, Zena et al. 2001) and mice with a mutation in the eIF2α phosphorylation site (Ser51Ala) (Scheuner, Song et al. 2001) that β-cells are particularly sensitive to ER stress induced dysfunction and death. Furthermore, studies in the Akita mouse showed that ER stress, secondary to mutated insulin can lead to β-cell death and glucose intolerance (Ogawa, Noma et al. 1995). Thus, while ER stress was previously known to be sufficient to induce β-cell dysfunction, we sought to provide the evidence that ER stress was induced under hyperglycemic conditions.

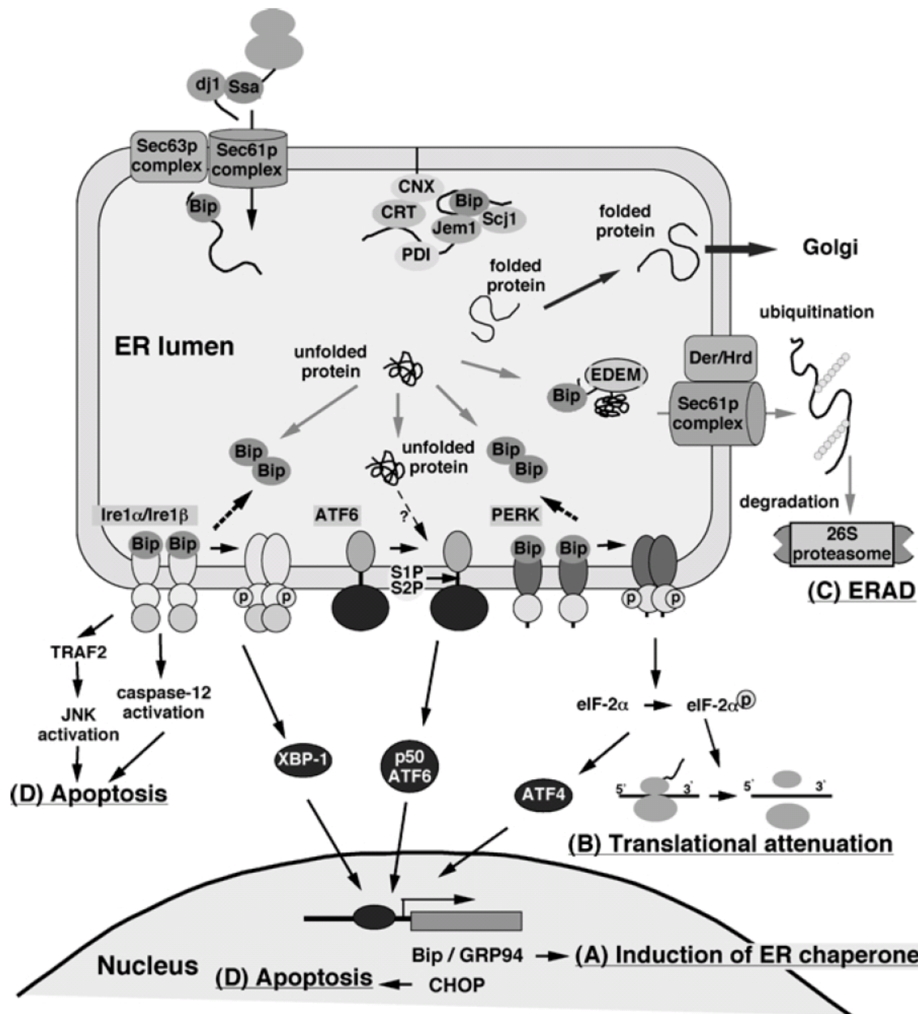


**Figure 4. A schematic representation of the unfolded protein response (UPR)**

P58 (= 58 kDa inhibitor of double stranded activated protein kinase PKR) is a negative feedback gens from nucleus inhibition of PERK (= PKR-like endoplasmic reticulum kinase) (not shown). GRP94 (= glucose-regulated protein 94) is an unfolded protein response chaperon as BIP (= immunoglobulin heavy chain binding protein), ATF4 (= activating transcription factor 4) is a transcription factor downstream PERK, EDEM (= ER degradation enchanching α-mannodiase-like protein) is a component of endoplasmic reticulum associated degradation. XBP-1 (transcription factor X-box binding protein 1) which is generated by IRE1 (= inositol requiring enzyme 1) -dependent XBP-1 mRNA splicing. When function of the ER is servely impaired, apoptosis is induced by transcription of CHOP (= C/EBP homologuos protein). (From the article: *Endoplasmic reticulum stress-mediated apoptosis in pancreatic beta-cells*, Oyadomari, Araki et al. 2002).

# 2 HYPOTHESIS

## 2.1 OVERALL HYPOTHESIS

Hyperglycaemia and cytokines lead to β-cell dedifferentiation and ER stress in pancreatic β-cells. This contributes to the loss of GSIS and increased apoptosis that underlies the patophysiology of type 2 diabetes.

## 2.2 SPECIFIC HYPOTHESES

**Hypothesis 1**: Hyperglycemia leads to ER stress in pancreatic β-cell and this contributes to increased apoptosis.
**Aim 1**: To examine the time course apoptosis of MIN6 cells treated with glucose.
**Aim 2**: To examine the expression of ER associated genes in MIN6 and islets of C57BL/6J and DBA/2 mouse treated with glucose
**Hypothesis 2**: The loss of β-cell phenotype (dedifferentiation) coincides with the loss of GSIS in cytokine-treated β-cells.
**Aim**: To examine the expression of β-cell differentiation genes in insulin-secreting MIN6 cells treated with cytokines.

## 2.3 Candidate genes

The choice of genes analysed in the course of these studies will be dictated by known functionality and on candidates identified in previous studies (unpublished data by Laybutt et al. 2005). By assessing a few key genes in each of a number of functional processes, we will obtain an accurate indication of the overall metabolic and differentiation state of the grafted β-cells. Genes that are highly expressed such as insulin, the islet-associated transcription factors and the multiple genes that confer the "glucose sensing" ability of the β-cell are down-regulated with dedifferentiation. In contrast, genes that are normally suppressed and would theoretically interfere with optimal β-cell functions are increased. β-cell glucose sensing genes refer to those involved in glucose metabolism (the glucose transporter, GLUT2; the rate-limiting enzyme of the glycerol-phosphate shuttle, mitochondrial glycerol phosphate dehydrogenase, mGPDH) and ion transport (Kir6.2, and voltage-

dependent $Ca^{2+}$ channels SERCA2b, etc). Transcription factors important for pancreas and islet development and the maintenance of β-cell differentiation include Nkx6.1, BETA2/NeuroD, PDX-1, Pax6 etc. These genes may be globally down-regulated or we may find heterogeneity among changes, which would suggest more important roles for specific genes.

The same methods will be use for the investigating in ER-stress associated genes. Results will determine whether the differentiation of β-cells and the UPR parallels, precedes or follows the loss of insulin secretory function.

The concentrations of glucose and cytokines used in these studies have widely been used previously in the literature to investigate the mechanisms responsible for hyperglycemia- and cytokine-induced β-cell dysfunction.

# 3 MATERIALS AND METHODS

## 3.1 Materials

Cytokines were obtained from R&D system, Inc, by Australia.All primers were obtained from Proligo, by Australia, Genset Pacific PTY. Ltd. The 100 basepair DNA ladder and the loading dye (6X) were obtained from Promega,Madison, by USA. Ethidium bromide was obtained from Amresco, by Australia. AmpliTaq Gold with GeneAmplification used for PCR was obtained from Roche, Applied Biosystems, by USA.
“High Pure purification” kit was obtained from Roche Diagnostics, Mannheim, by Germany.
Superscript First-Strand Synthesis system for RT-PCR was obtained from Invitrogen, Life technologies, by Australia. Lightcycler FastStart DNA Master SYBR Green 1 and lightcycler capillaries were obtained from Roche, Diagnostics GmBH, by Germany. Thermo tubes 0.2 ml was obtained from ABgene, Advanced Biotechnologies Ltd. Micro titre plates with 96 wells were obtained from Nunc, by Denmark. 50 ml polypropylene conical tubes were purchased from Falcon, by USA. Aerosol barrier tips 20 E were obtained from Promega. Barrier tips 10 E were obtained from CLP, by Australia.
RNeasy Mini Kit for total RNA isolation from animal cells was obtained from Quigen, by Australia.
Rat Insulin RIA Kit was obtained from Linco Reserch, Inc. by Australia. Pst1 restrictions enzymes were obtained from Promega, by Australia.

## 3.2 Animals

C57BL/6J and DBA/2 mice were taken from the Garvan Institute Biological testing Facility, were kept under conventional conditions with free access to water and standard laboratory chow.
All animal procedures were approved by the Animal Experimentation Ethics Committee (AEEC) at Garvan Institute of Medical Research/ St.Vincent’s hospital, Australia.

## 3.3 Cells

MIN6 derived from mouse insulinoma. They maintain a high state of β-cell differentiation. They secrete insulin in a glucose responsive manner. Islets are balls of several different cell types. Therefore, changes in gene expression may be influenced by changes in several different cell types. We can use MIN6 cells to study β-cells in isolation.

## 3.4 Methods

### 3.4.1 Pancreas preparation and islet isolation

Separate pancreas were exised after liberase injection into the common pancreatic duct and put into flasks, containing islet medium (with a final concentration of 1* X-199, $NaHCO_3$ 14 mM, NCS 10 %, penstrep 500 iU+100 µg/ml, gentamycin 50 µg/ml, glucose 0.5 µg/ml, $H_2O$ and glutamine 2 mM). The flasks were rinsed with 15 ml of warm KRB buffer (containing 50 % KRB stock (2X), hepes 1 M, $CaCl_2$ 100 mM, $H_2O$ and glucose 2.8 mM) and then poured to 10 ml. Next step was incubation for 20 minutes at 37°C with the digestive enzyme liberase. After incubation, cold KRB was added up to 40 ml to stop the incubation. The flasks were inverted twice and then settled for 5 minutes on ice. After removal of fat, the contents were dispersed with a 10 ml syringe once, pipetted with 5 ml Gilson pipette 5 times and then returned to the flasks. They were then centrifuged for 2 seconds at 600 rpm, the supernatant was poured off and 10 ml KRB was added. The previous step was repeated 3 times. Next the media was poured through a sieve and returned to clean flasks. To each old flask, 15 ml of KRB added and rinsed through the sieve so the total volume per flask was 25 ml. The flasks were centrifuged at 1500 rpm for 10 seconds and decanted. Then 20 ml of cold Histopaque (containing Ficoll Type 400, distilled water and sodium diatrizoate) was added and vortexed until the suspension was homogenous. Thereafter, KRB was layered on the top of the buffer and the separate flasks were centrifuged at 1900 rpm for 20 minutes. The interface between the layers was removed to a new flask and KRB was added to 40 ml, centrifuged at 1500 rpm for 10 seconds and the supernatant removed. As the last step 40 ml of KRB was added to each flask and then transferred to a dish to be handpicked under a stereomicroscope to ensure a pure islet preparation. The number of islets varied between 100-200 per mouse.

### 3.4.2 Cell culture and treatment

MIN6 cells were passaged in 150 $cm^2$ flask with 25 ml Dulbeccos modified Eagles medium(DMEM invitrogen , Carsbad, CA) containing 25 mM glucose media added with 1 % streptomycin penicillin, 0,75 % Hepes, 10 % FCS and 5 % serum. All MIN6 cells used in experiments were between passages 29 and 38.

### 3.4.3 Seeding MIN6 cells for mRNA

Cells were incubated in DMEM. Warm PBS (Phosphate-buffered saline) were added for washing cells, then 5 ml trypsin was added to loosened up cells from flask, then sucked off and incubated at 37°C for 2-3 minutes. After that 10 ml glucose media was added, cells were split and seed in either 12- or 24-wells plates containing $3x10^5$ - $5x10^5$ cells/well.

Cytokine treatment in MIN6 cells: cells were treated with either IL1-β (0.5 ng/ml) or a cocktail of IL1-β (0.5 ng/ml), TNF-α (6.25 ng/ml) and IFN-γ (75 ng/ml) in 0.5 ml DMEM media per well. Then incubated at 37°C for 24-48 hours.

High and low glucose treatment in MIN6 cells: cells were treated with 0.5 ml DMEM media with 5 mM, 25 mM or 40 mM glucose for 4, 24, 48 and 72 hours. The media were changed every 24 hours.

### 3.4.4 Extraction of total RNA from the cells

RNA was extracted from the isolated islets and MIN6 cells according to the RNeasy RNA extraction protocol (Qiagen). Briefly, cells were disrupted when added to the buffer RTL. Then 350 µl RLT buffer was added to each tube and then vortexed. The next step was to homogenize the sample by passing the lysate 5 times through a 0.9 mm needle fitted to an RNase-free syringe. For each tube, 70 % ethanol and was added to the homogenized lysate. Each tube was mixed well by pipetting. The samples were applied separately to RNeasy mini columns and placed in 2 ml collection tubes. The columns were centrifuged for 15 seconds at 13000 rpm and the flowthrough discarded. Subsequently, a washing buffer was added to each RNeasy column and centrifuged for 15 seconds at 13000 rpm. The flowthrough and the collection tubes were discarded. The RNeasy columns were transferred into new 2 ml collection tubes and 500 µl Buffer RPE pipetted into the RNeasy column. The tubes were centrifuged for 15 seconds at 13000 rpm to wash the columns again. 500 µl Buffer RPE was again added to the columns and centrifuged for 2 minutes at the same speed as previous mention to dry the RNeasy silicagel membrane. Then, the samples were eluted by adding 20 µl RNase-free water directly into the RNeasy silica-gel membrane and centrifuged for 1 minute at 13000 rpm. The RNA samples were stored at -80 °C.

### 3.4.5 RNA Quantification

RNA quantification was performed with a Beckman Spectrophotometer at 260 nm. For measuring, the stock RNA was diluted with distilled water to 1:100 dilution.

### 3.4.6 Reverse transcriptase (RT) reaction

mRNA was copied using reverse transcriptase enzyme to synthesize cDNA (SuperScript First-Strand Synthesis for RT-PCR). The components per micro tube were: 0.1 µg RNA, 1 µl 10 mM dNTP mix, 1 µl Oligo (dT) 12-18 (0.5 µg/µl) to a total of 10 µl. The samples were incubated for 5 minutes at 65°C and then put on ice. Then a mix containing 20 % 10* RT buffer, 40 % 25 mM $MgCl_2$, 20 % 0.1 M DTT, 10 % RNaseOUT and finally 10 % Superscript ll RT. Each tube was heated for 50 minutes at 42°C and 15 minutes at 70°C. To the reaction mix, 1 µl of RNase H was added and incubated for 20 minutes at 37°C to remove residues of mRNA.

### 3.4.7 Insulin secretion from MIN6 mice

Preparing for insulin secretion assay (RIA):
Krebs-Ringer buffer (KRB) was made by mix of KRB, 1 % HEPES 1M, 1 % $CaCl_2$ 100 mM, $H_2O$ and glucose. The mix was gassed with $CO_2$ for 15 minutes and Bovostar (BSA, bovine serum albumin) 0.25 % was added. KRHB with 2.8 mM glucose were prewarm and 100 µl of the DMEM media was added to a 96-well plate. Cells were washed with 1 ml KRHB 2.8 mM glucose, and then pre-incubated for 1 hours at 37°C in 0.5 µl of KRHB with 2.8 mM or 16.8 mM glucose.
Samples were then put on ice and KRHB was transferred to the 96-well plate. Cells were washed 3 times with cold PBS (Phosphate-buffered saline) discharge and 500 µl ice cold water was added for lysis the cells. Cells were then scraped and transferred to 1.5 ml eppendorf, sonicated and stored in the freezer until day for assay.

Lincos Rat insulin RIA kit utiliszes an antibody made specifically against rat insulin. Sensitivity of 0.1 ng/ml pr. 100 µl serum sample in an overnight equilibrium assay. This kit was used to compare the insulin secretion of MIN6 cell after treated with cytokines for 24-48 hours to the control.
The protocol was followed with some volumes changes. The standard curve was made by adding 50 µl of assay buffer to the non-spesific binding (NSB) tubes and 25 µl of buffer to reference tubes. 25 µl of standards and quality controls, 25 µl of sample, 25 µl of I-insulin label to all tubes, 25 µl of rat antibody to all tubes except totals and NSB. Tubes were vortex, cover and incubate overnight at 4 degree.
Next morning 250 µl of precipitating reagent and 2 ml extra precipitating reagent (PBS, 25 mM EDTA, 1 % BSA) were added to all tubes(except totals). Then vortex and incubate for 20 minutes at 4°C, after that tubes (except totals) were centrifuge for 40 minutes at 3200 x g at 10°C. Supernatant were decadent, tubes were drained for 1 minute and Insulin concentration was counted at Wallac gamma counter.

### 3.4.8 Polymer-chain-reaction (PCR)

**Testing of primers**

Testing of the primers was done using the polymer chain reaction (PCR). Stock solution of the primer pairs was diluted with distilled water to a concentration of 30 µM. The components of the Master Mix for each tube were 5 µl Taq Pol Buffer, 3 µl 1.5 mM $MgCl_2$ , 1 µl dNTP, 0.5 µl forward primer, 0.5 µl reverse primer, 1 µl AmpiTaq Gold and distilled water up to 48 µl and finally 2 µl of cDNA was added to each tube.
The PCR was running for 40 cycles with a cycle of 30 seconds for each step, 72°C, 55°C and 95°C. Thereafter, gel electrophoresis was run to check that the primers produced a single band at the correct size. For each tube containing 10 µl of the reaction mix above, 2 µl of 6X Buffer Loading Dye was

added to each tube and mixed well. The individual samples were loaded on a 1.2 % agarose gel run on TAE-buffer and stained with ethidium bromide. The gel containing the nucleic acids was examined under UV light and the products compared with a molecular weight marker (100 bp DNA ladder).

### 3.4.9 Gene size chart

| Gene | Size | Forward primer sequence | Reverse primer sequence |
|---|---|---|---|
| ATF4 | 217 | 5'- ATCCAGCAAAGCCCCACAAC -3' | 5'- CAAGCCATCATCCATAGCCG -3' |
| BETA2/NeuroD | 276 | 5'-ACTCCAAGACCCAGAAACTGTC-3' | 5'-ACTGGTAGGAGTAGGGATGCAC-3' |
| BiP-GRP78 | 262 | 5'- AGGACAAGAAGGAGGATGTGGG -3' | 5'- ACCGAAGGGTCATTCCAAGTG -3' |
| CHOP-10/GADD153 | 176 | 5'- TTCACTACTCTTGACCCTGCGTC -3' | 5'- CACTGACCACTCTGTTTCCGTTTC -3' |
| Cyclophilin | 151 | 5'-TGTGCCAGGGTGGTGACTTTAC-3' | 5'-TGGGAACCGTTTGTGTTTGG-3' |
| EDEM1 | 157 | 5'- GCAATGAAGGAGAAGGAGACCC -3' | 5'- TAGAAGGCGTGTAGGCAGATGG -3' |
| Erp72 | 245 | 5'- AGTCAAGGTGGTGGTGGGAAAG -3' | 5'- TGGGAGCAAAATAGATGGTAGGG -3' |
| GLUT2 | 221 | 5'-CATTCTTTGGTGGGTGGC-3' | 5'-CCTGAGTGTGTTTGGAGCG-3' |
| ID-1 | 210 | 5'-TTGGTCTGTCGGAGCAAAGC-3' | 5'-GCAGGTCCCTGATGTAGTCGATTAC-3' |
| iNOS | 519 | 5'- GCACCTTGGAAGAGGAGCAACTAC -3' | 5'- TGCGGCTGGACTTTTCACTC -3' |
| IRS2 | 280 | GCAACACACCCGAGTCAATAGC | AGGCGACCTGAACTACCAGAGAAG |
| Kir6.2 | 261 | 5'-TCGTGTCCAAGAAAGGCAACTG-3' | 5'-GGAAGGCAGATGAAAAGGAGTGG-3' |
| mGPDH | 206 | 5'- AAAGACTGGAGCCCCACACTCTAC -3' | 5'- ATCCCGTATTTCACCTCTGCTTC -3' |
| Nkx6.1 | 212 | 5'-GGACCAGAGAGAGCACGC-3' | 5'-TTCGGGTCCAGAGGTTTG-3' |
| p58 | 160 | 5'- AAGCCCGTGGAAGCCATTAG -3' | 5'- GGTCATTTTCATTGTGCTCCTGAG -3' |
| Pax-6 | 178 | 5'- TGCCCTTCCATCTTTGCTTG -3' | 5'- TCTGCCCGTTCAACATCCTTAG -3' |
| PDX-1 | 172 | 5'-CGGACATCTCCCCATACG-3' | 5'-AAAGGGAGCTGGACGCGG-3' |
| SERCA2b | 259 | 5'- TGGAGACAACAAAGGCACCG -3' | 5'- CAGAGCAGGAGCATCATTCACAC -3' |
| Xbp-1 | 269 | 5'- GCAGCAAGTGGTGGATTTGG -3' | 5'- AGATGTTCTGGGGAGGTGACAAC -3' |

### 3.4.10 PCR Standard preparation

PCR was performed for each gene to prepare standards for Real-Time-PCR. The same reaction mix was made and the same cycling conditions were applied as described above for the testing of primers.
The PCR product was purified using a “High pure PCR Product Purification Kit”. The manufacturer’s instructions were followed, by adding 1 ml binding buffer to each reaction and mixing well. The samples were transferred to the upper reservoir of a High Pure filter tube in a collection tube. The collection tubes were centrifuged at 45 seconds at maximum speed in a standard bench top centrifuge at room temperature. The filter tubes were disconnected and the flowthrough solution was discharged and re-connected to the same collection tube. Further, 500 µl washing buffer was added to the upper reservoir and centrifuged for 1 minute at maximum speed. The flowthrough solution was discharged and 200 µl of washing buffer was added again to ensure optimal purity. Both the collection tubes and the flowthrough solution were discarded and the filter tubes were re-connected with a clean microcentrifuge

tube. Elution buffer of 100 µl was added to the upper reservoir of the filter tube and centrifuged for 1 minute at maximum speed. The purified DNA was stored at -20°C.

### 3.4.11 Real-time polymer-chain-reaction (RT-PCR)

Real-time PCR was undertaken on a Lightcycler (Roche) using a commercial reagent kit (Lightcycler - FastStart DNA Master SYBR Green l (Roche)). According to manufacturers instructions, a mixture containing 0.6 µl 1.5 mM $MgCl_2$, 0.2 µl forward primer, 0.2 µl reverse primer, 0.5 µl HotStart enzyme, 1 µl cDNA, 7.5 µl $H_2O$ to a total of 10 µl for each tube. 10 µl was added to glass capillaries tubes and transferred to the Roche Molecular Biochemical Lightcycler rotor. The thermal cycle profile employed a 10 minutes denaturation step at 95°C followed by an amplification step of 40 cycles (15 seconds at 95°C, 5 seconds at 55°C and 10 seconds at 72°C).
A standard curve was made for each gene using serial 1:10 dilutions. The housekeeping gene, Cyclophilin was used as the control gene to correct for experimental variations between samples. The arbitrary values of the samples were so calculated from the standard curve and concentrations of the samples were calculated by comparing the DBA/2 mice expressed relative to C57BL/6 mice in 11.1 mM glucose mice, which were set to 100 %. In the glucose concentration treatment 25 mM and 40 mM are expressed relative to the 5 mM values which are set at 100 % for each time point. And cytokines treatment expressed relative to the control, which was set to 100 %.

All the RT-PCR products were tested on a 1.2 % agarose gel using a TAE buffer containing 10 % TAE (Tris-acetate 4.84 % (40 mM), 1.15 % glacial acetic acid, 2 % EDTA (0.5 M)). A 100 base par DNA ladder was used as a marker. The gel was stained with ethidium bromide. Finally the gel was visualized on a Bio-RAD GEL 1000 spectrophotometer by using UV light. The fragment parts were checked for the correct fragment size.

### 3.4.12 XBP-1 processing

To detect XBP-1 gene by PCR and gel electrophoresis: Stock solution of the primers pairs were diluted as above in testing the primer. The components of the Master Mix for each tube were 2.5 µl Tag Pol Buffer, 1.5 µl 25mM $MgCl_2$, 0.5 µl dNTP 10 µM, 0.5 µl of each primer. 18 µl dd$H_2O$ and 0.5 µl Apmli TagGold, and 1 µl of cDNA. The PCR was running for 35 cycles with a cycle of 30 seconds for each step, 94°C, 55°C, and 72°C.Then the restriction enzymes Pst1 was added and digested for 5 hours at 37°C.
Thereafter 7 µl of Gel loading buffer were added and run on gel electrophoresis. 1- 1.2 % agarosa-gel was used on a TAE-buffer and stained with ethidium bromide. The gel was examined under UV light and the product was compared with a molecular weight marker (100 bp DNA ladder). Cut and uncut

(active component) fragments, were compared with percentage of the total, the intensity of gene on gel were measured to percentages adjusted volume.

### 3.4.13 Apoptosis assay on FACS

Ethanol fixation of cells: cells were harvested by trysination and centrifuged down at 1000 rpm for 3 minutes. Then washed with PBS and centrifuged down again at 1000 rpm for 3 minutes. Tubes were hold at 45°C and very slow, dropwise add 1 ml/1x$10^6$ cells of cold 80 % ETOH while vortexing gently. Then cells were centrifuged at 1000 rpm for 5 minutes, ETOH were discharged and 0.5 ml BS/tween added before pripidium Iodide (PI) staining;
PI was added (from stock 1 mg/ml = 0.1 %) 10 µl /ml of cell solution. Then added RNAse A (stock 10 mg/ml at 50 µl/ml of cell solution). Cells were so incubated for at least 1 hour at room temperature in the dark. Samples were run on Fluorescence Activated Cell Sorting (FACS).

**Presentation of data**
The results are expressed as the means ± SEM from two experiments performed in triplicate.

# 4 RESULTS

In this project, islets isolated from two different mouse strains were utilized for studying the mechanisms responsible for β-cell dysfunction. Knowledge of changes in islet gene expression due to chronic exposure of islets to high glucose levels characteristic of the diabetic state have not been extensively documented. In addition to studies with animal islets, experiments were performed using MIN6 cells as a pure β-cell model, for characterizing the changes in gene expression, insulin secretion and apoptosis due to chronic exposure to high glucose and cytokines.

The Results will be presented in two parts:

**Part 1** presents studies investigating the regulation by glucose of edoplasmic reticulum (ER) stress genes and apoptosis in MIN6 cells and mouse islets.

**Part 2** presents studies investigating the regulation by cytokines of β-cell differentiation and insulin secretion in MIN6 cells.

## 4.1 Part 1: Glucose regulation of ER stress and apoptosis in β-cells

### 4.1.1 Time Course changes in apoptosis in MIN6 cells exposed to different concentrations of glucose

Long-term culture of MIN6 cells in different concentrations of glucose produced changes in the rate of apoptosis. As expected, the percentage of apoptotic cells was increased with time of exposure regardless of the level of glucose. All three concentrations of glucose; 5 mM, 25 mM and 40 mM, are low, normal, and high respectively for MIN6 cells (Busch, Cordery et al. 2002) showed higher apoptosis at 72 h compared to 48 h (figure 4.1). The level of apoptosis was lowest in cells cultured at 25 mM glucose consistent with the fact that this is the normal basal level of glucose in culture media for MIN6 cells. A modest increase in apoptosis was observed in cells cultured at 40 mM glucose compared to 25 mM glucose. This indicates that the higher glucose levels were slightly toxic to the MIN6 cells, but that predominantly the cells were somehow protected from large increases in apoptosis. Surprisingly, the marked increase in apoptosis occurred in cells cultured at low glucose (5 mM) compared to the other groups. These intriguing findings raise two questions:

1) what are the mechanisms responsible for protecting MIN6 cells from overt increases in apoptosis due to high glucose, and 2) what are the mechanisms responsible for increased apoptosis in cells cultured at low glucose levels?

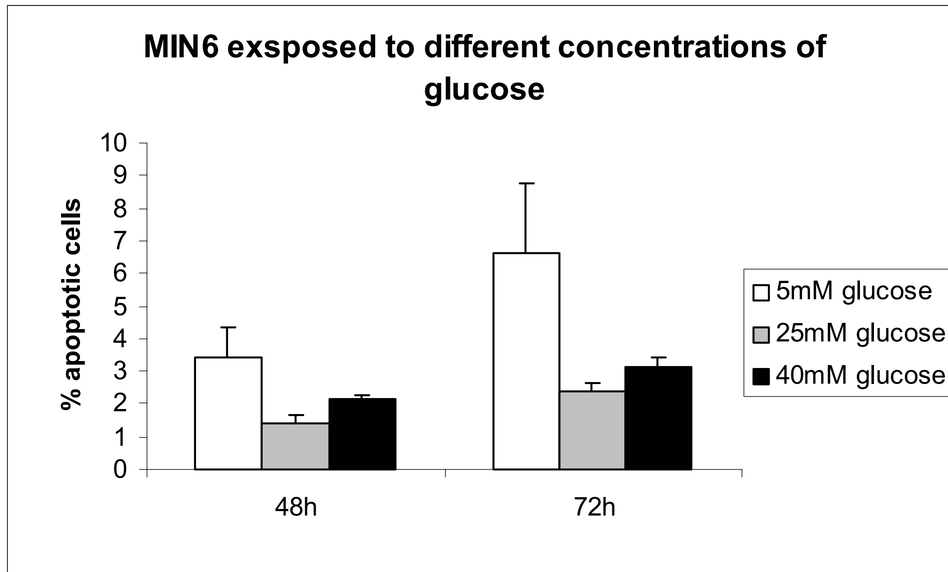


Fig 4.1. Time course changes in apoptosis in MIN6 cells exposed to different concentrations of glucose. MIN6 cells were treated for 48 or 72 h with either 5, 25 or 40 mM glucose, and apoptosis measured using FACS. Expressed as percentage of total cells. The values are means ± SEM, (n=2).

### 4.1.2 Time course changes in ER stress gene mRNA levels in MIN6 cells exposed to different concentrations of glucose

Conditions that disrupt endoplasmic reticulum (ER) functions, termed ER stress, lead to the accumulation of misfolded proteins in the ER. This triggers a signalling program known as the unfolded protein response (UPR) that facilitates attenuation of the stress or in the case of severe stress leads to apoptosis. In this project, we cultured MIN6 cells at different glucose levels and tested for the presence of ER stress. We measured changes in ERstress associated genes in MIN6 cells cultured at 5, 25 and 40 mM glucose.

The time-dependent changes in mRNA levels of several ER stress genes in MIN6 cells were examined by real-time RT-PCR (oligonucleotide primer sequences specific for each gene tested are listed in materials and methods section). To investigate deviations from the normal pattern of β-cell gene expression, mRNA levels of each gene in question were normalized for a control gene (Cyclophilin). The value obtained for each specific product was expressed relative to Cyclophilin for each sample (ratio of specific product/Cyclophilin). These ratios were then expressed as a percent of the ratio in control cell extracts which was set at 100 % at each time point.

When functions of the ER are severely impaired, apoptosis is induced by transcriptional activation of the gene for C/EBP homologous protein (CHOP). However, it is important to note that CHOP induction is also a feature of the so-called integrated stress response induced. Figure 4.2 shows time- and glucose- dependent changes in CHOP mRNA levels. CHOP mRNA levels were highest in cells treated with low (5 mM) glucose concentrations. Furthermore, the difference in CHOP mRNA levels between the 5 mM glucose groups and the groups with higher concentrations of glucose was accentuated over time. We therefore observed that the increased CHOP mRNA levels in cells with low glucose concentrations are associated with the highest levels of apoptosis (Figure 4.1).

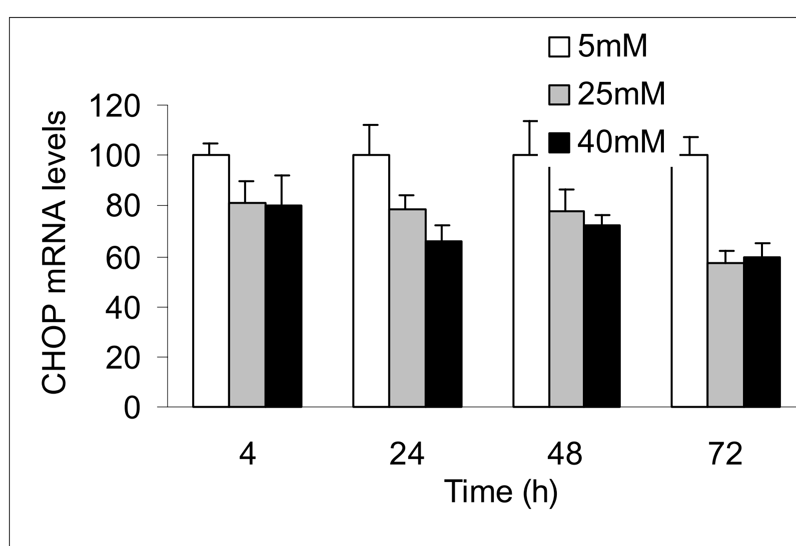


Fig 4.2. mRNA levels of the apoptosis-associated gene C/EBP homologous protein (CHOP) in MIN6 cells expressed as a percentage of 5 mM set at 100 % at each time point. The mRNA level was measured at 4, 24, 48 and 72 hours. The values are means ± SEM, (n=2).

In contrast to CHOP, which can be induced by both ER stress and the integrated stress response, up-regulation of several genes associated selectively with ER stress were found in MIN6 cells exposed to high glucose (40 mM) concentration. Immunoglobulin heavy chain binding protein (BiP) serves as an ER chaperone and a sensor of protein misfolding, and its up-regulation is characteristic and indicative of ER stress. BiP mRNA levels were increased by glucose in a time-dependent manner (Table 4.1): BiP mRNA levels were markedly induced in cells exposed to
40 mM glucose at 48 and 72 h. Similarly, mRNA levels for the ER protein disulfide isomerase, ERP72 were induced in cells exposed to 40 mM glucose at 48 and 72 h (Table 4.1). Up-regulation of ER degradation enchanching α-mannodiase-like protein 1 (EDEM1), indicative of the ER-associated degradation response was also observed with high glucose levels in MIN6 cells. mRNA

levels of 58 kDa inhibitor of double stranded activated protein kinase PKR (p58), another ER stress protein induced predominately downstream of ER stress sensor genes and X-box binding protein 1 (XBP-1), were unchanged at 4 and 24 h, but increased at 48 and 72 h, displaying a time-dependent effect. In contrast, levels of activating transcription factor 4 (ATF4) mRNA, known to be induced downstream of eukaryotic translation initiation factor 2α (eIF2α) phosphorylation, were either unchanged or slightly reduced in the high glucose groups. eIF2α is a substrate for other stress-activated protein kinases so its activation, and the activation of ATF4 and CHOP, are features of the integrated stress response.

Table 4.1.

mRNA level of genes involved in the unfolded protein response of the endoplasmic reticulum. The mRNA levels in the MIN6 cells exposed to glucose are compared to the 5 mM at each time point (set at 100 %). Values are means ± SEM, (n=2). (see section 4.1.2). P58 = 58 kDa inhibitor of double stranded activated protein kinase PKR; ATF4 = activating transcription factor 4; ERP72 = endoplasmic reticulum protein 72; BiP = immunoglobulin heavy chain binding protein; EDEM1 = ER degradation enchanching α-mannodiase-like protein 1.

| Gene | Glucose | 4 hours | 24 hours | 48 hours | 72 hours |
|---|---|---|---|---|---|
| P58 | 5 mM | 100±25 | 100±14 | 100±15 | 100±3 |
| | 25 mM | 93±6 | 123±14 | 161±19 | 156±29 |
| | 40 mM | 81±7 | 131±7 | 179±42 | 211±37 |
| | | | | | |
| ATF4 | 5 mM | 100±29 | 100±19 | 100±5 | 100±13 |
| | 25 mM | 69±7 | 88±15 | 97±14 | 77±8 |
| | 40 mM | 88±33 | 74±9 | 98±19 | 92±6 |
| | | | | | |
| ERP72 | 5 mM | 100±3 | 100±11 | 100±12 | 100±7 |
| | 25 mM | 78±2 | 93±11 | 133±19 | 110±23 |
| | 40 mM | 83±12 | 84±7 | 165±37 | 174±54 |
| | | | | | |
| BiP | 5 mM | 100±12 | 100±15 | 100±13 | 100±17 |
| | 25 mM | 67±7 | 106±26 | 153±21 | 101±29 |
| | 40 mM | 62±9 | 145±18 | 250±13 | 221±64 |
| | | | | | |
| EDEM1 | 5 mM | 100±19 | 100±9 | 100±5 | 100±11 |
| | 25 mM | 122±6 | 91±12 | 175±36 | 121±27 |
| | 40 mM | 123±2 | 100±8 | 190±14 | 186±37 |

### 4.1.3 Change in processed XBP-1 in MIN6 cells

Activation of the UPR transducer protein, inositol requiring enzyme 1 (IRE1) leads to splicing of X-box binding protein 1 (XBP-1) mRNA. Thus, to differentiate ER stress responses from the integrated stress response we took advantage of the fact that ER stress induced splicing of XBP-1 mRNA results in a frame-shift through which there is a rearrangement to an active form and the loss of a Pst1 restriction site. We therefore examined XBP-1 activation in MIN6 cells exposed to different glucose levels by PCR amplifying XBP-1 cDNA followed by incubation with Pst1. The processed mRNA level of XBP-1 was found to be up-regulated in high glucose concentration (Fig. 4.3), indicating an increase in the active form of XBP-1 mRNA and the presence of ER stress. These data provide evidence of UPR activation and the presence of ER stress in MIN6 cells exposed to high glucose levels.

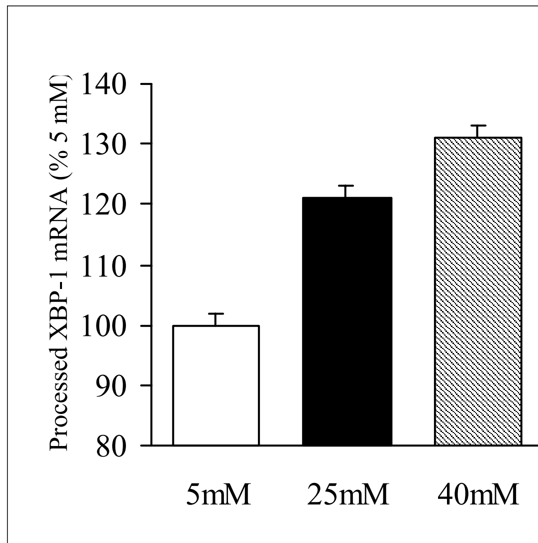


Fig 4.3. Altered X-box binding protein 1 (XBP-1) splicing in MIN6 cells exposed to varying glucose levels. RNA extracted from MIN6 cells was reverse transcribed. XBP-1 cDNA was amplified by PCR and digested with Pst1, which cuts unprocessed XBP-1 cDNA into fragments. Processed (activated) XBP-1 cDNA lacks the restriction site and remains intact. Processed (intact) and unprocessed (cut) XBP-1 was quantified by densitometry. The value obtained for processed (activated) XBP-1 was expressed as a ratio of the total (processed + unprocessed) XBP-1 mRNA levels for each experiment. These ratios are expressed as a percentage of the ratio in MIN6 cells cultured at 5 mM (set at 100 %) glucose after 24 h exposure. The values are means ± SEM, (n=2).

To summarise these data, in the case of **high glucose** levels, ER stress is induced with activation of the protective aspects of the UPR, including increased ER chaperones, folding enzymes and misfolded protein degradation pathways (BiP, ERP72, EDEM). Thus, ER stress induced by high glucose provides protection from apoptosis. On the other hand, **low glucose** levels induce apoptosis and CHOP mRNA levels in MIN6 cells. This is probably mediated by the integrated stress response, independent of ER stress.

### 4.1.4 Time course change in transcription genes mRNA level MIN6 cells exposed to different concentrations of glucose

Transcription factors provide the genetic instructions that drive pancreatic development and enable mature β-cell to function properly. Mutation in several transcription factors involved in the pancreatic transcriptional network have been linked to the Maturity Onset Diabetes in the Young (MODY). Disruption of this specialized set of transcription factors have been proposed to contribute to the development of insulin secretory dysfunction.

The transcription factor NK6 transcription factor related, locus 1 (Nkx6.1) has been shown to be important for β-cell differentiation. Over the time course and range of glucose levels used in these studies there was relatively little change in the mRNA level of Nkx6.1 (Figure 4.4). This indicates that the treatments used in the course of these experiments were well tolerated by the cells without significant adverse influence on their differentiation state.

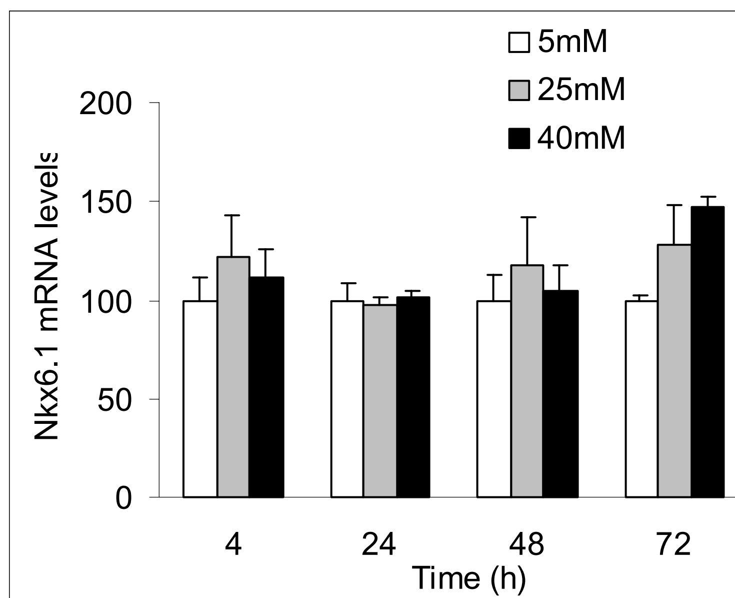


Fig. 4.4. mRNA level of the transcription factor NK6 transcription factor related, locus 1 (NKX6.1) in MIN6 cells exposed to 5 mM, 25 mM and 40 mM glucose expressed as a percentage of 5 mM (set at 100 %) at each time point. The mRNA level was measured at 4, 24, 48, and 72 hours. The values are means ± SEM, (n=2).

Insulin receptor substrate 2 (IRS-2) plays a critical role in β-cells. Increased IRS-2 expression promotes β-cell growth and survival, whereas decreased IRS-2 levels leads to apoptosis. mRNA levels of IRS-2 showed an increase in high glucose concentration (Figure 4.5). This reinforces the notion that high glucose promotes growth in MIN6 cells without increases in apoptosis.

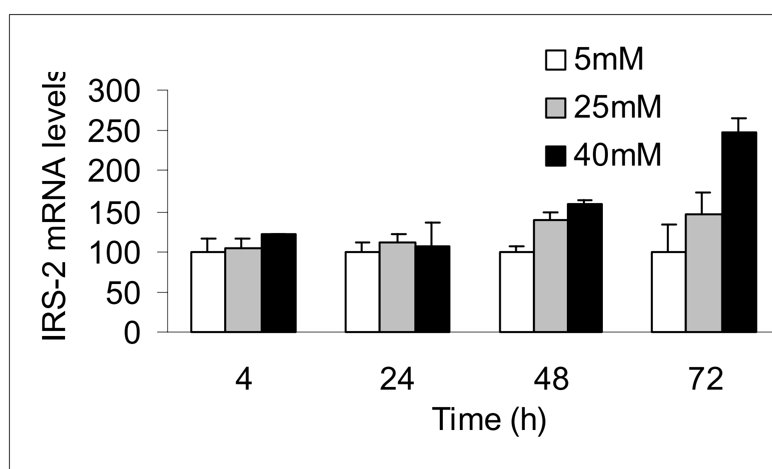


Fig 4.5. mRNA level of the insulin substrate gene Insulin receptor substrate 2 (IRS-2) in the MIN6 cells exposed to 5 mM, 25 mM and 40 mM glucose expressed as a percentage of the 5 mM (set at 100 %) at each time point. The mRNA level was measured at 4, 24, 48 and 72 hours. The values are means ± SEM, (n=2).

### 4.1.5 Regulation of ER stress gene expression in mouse islets

We next examined the regulation by glucose of ER stress gene expression in whole isolated islets from mice. Since there are recognized differences in gene expression of mouse islets from various strains, we tested the regulation of ER stress genes in isolated islets from two different mouse strains: C57BL/6 and DBA/2.

### 4.1.6 Change in ER-stress associated mRNA level in mouse islets exposed to different concentrations of glucose

Islets were isolated from the mice and pre-cultured overnight (in RPMI 1640 medium containing 10 mM glucose and 10 % FCS) to allow islets to recover from the isolation procedure, and to eliminate islets with signs of central necrosis (usually large islets). The healthy cultured islets were used to determine whether the changes gene expression indicative of ER stress. The experimental conditions consisted of 48 h culture at 11, 30 and 40 mM glucose followed by RNA extraction and analysis of gene expression by real-time RT-PCR.

C/EBP homologous protein (CHOP) mRNA levels were lower in islets exposed to high glucose for 48 h. In other words, CHOP was induced by exposure of islets to low glucose levels, similar to the findings in MIN6 cells. Islet ATF4 mRNA levels showed a similar trend with glucose exposure (Table 4.2). Thus, the integrated stress response downstream of eukaryotic translation initiation factor 2α (eIF2α) phosphorylation (ATF4 and CHOP) shows induction in low glucose in mouse islets. ER-stress associated genes (BiP, EDEM1 and P58) were observed to increase in islets exposed to high glucose concentration (40 mM) compared to the lower glucose concentration [11mM, which is the normal glucose concentration for culturing islets (Zraika, Aston-Mourney et al. 2006)]. There were no differences in the regulation of these genes in islets from C57BL/6 versus DBA/2 mice (Table 4.2). The processed mRNA level of XBP-1 was found to be up-regulated in islets exposed to high glucose concentration (Fig. 4.6), indicating an increase in the active form of XBP-1 mRNA and the presence of ER stress.

Table 4.2.
mRNA level of genes associated in ER-stress in the islets isolated from C57/BL6 and DBA/2 mouse. The mRNA level is compared to the 11 mM glucose C57/BL6 mice (set at 100 %). Values are means ± SEM, (n=2).

| | C57/BL6 | | DBA/2 | | |
|---|---|---|---|---|---|
| Gene | 11 mM | 40 mM | 11 mM | 30 mM | 40 mM |
| CHOP | 100±23 | 13±2 | 85±6 | 14±1 | 13±1 |
| ATF4 | 100±61 | 58±12 | 139±36 | 73±16 | 58±16 |
| ERP72 | 100±34 | 100±34 | 92±19 | 105±7 | 114±16 |
| BiP | 100±15 | 246±12 | 122±17 | 128±46 | 155±28 |
| EDEM1 | 100±20 | 281±113 | 87±18 | 99±16 | 186±52 |
| P58 | 100±23 | 294±93 | 96±16 | 240±26 | 327±19 |

CHOP = C/EBP homologous protein; ATF4 = activating transcription factor 4; ERP72 = endoplasmic reticulum protein 72; BiP = immunoglobulin heavy chain binding protein; EDEM1 = ER degradation enchanching α-mannodiase-like protein 1; P58 = 58 kDa inhibitor of double stranded activated protein kinase PKR.

### Changes in processed XBP-1 gene in mouse islets

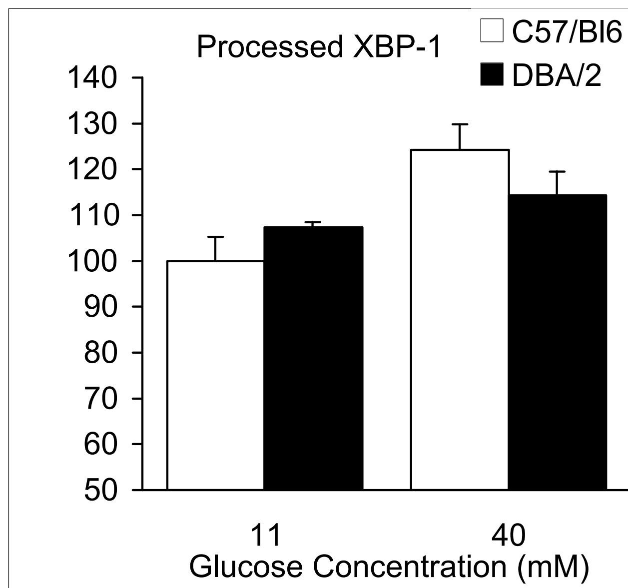


Fig 4.6. mRNA level of the ER-stress gene X-box binding protein 1 (XBP-1) in C57/BL6 and DBA/2 mouse islets after 48 hours exposed to glucose. RNA extracted from islets isolated from C57/BL6 and DBA/2 mice was reverse transcribed. XBP-1 cDNA was amplified by PCR and digested with Pst1 which cuts unprocessed XBP-1 cDNA into fragments. Processed (activated) XBP-1 cDNA lacks the restriction site and remains intact. Processed (intact) and unprocessed (cut) XBP-1 was quantified by densitometry. The value obtained for processed XBP-1 was expressed as a ratio of the total (processed + unprocessed) XBP-1 mRNA levels for each animal. These ratios are expressed as a percentage of the ratio in C57/BL6 islets at 11 mM set at 100 %. The values are means ± SEM, (n=2).

## 4.2 PART 2

### 4.2.1 Cytokine regulation of β-cell differentiation and function in MIN6 cells

Cytokines has been proven to play an important role in β-cell dysfunction and death, but the mechanism responsible has not been fully characterized. In this project, we have focused on the regulation by cytokines of β-cell insulin secretion, and the expression of insulin transcription genes and genes involved in the insulin secretion.

### 4.2.2 Changes in acute glucose induced insulin secretion in MIN6 cells exposed to cytokines

Figure 4.7 shows that MIN6 cells exposed to cytokines led to reduction in the acute stimulatory effect of glucose on insulin secretion compare to the control cells. At 48 h, a mixture of IL-1β, IFN-γ and TNF-α (cocktail) lead to a further reduction in secreted insulin compare to IL-1β alone.

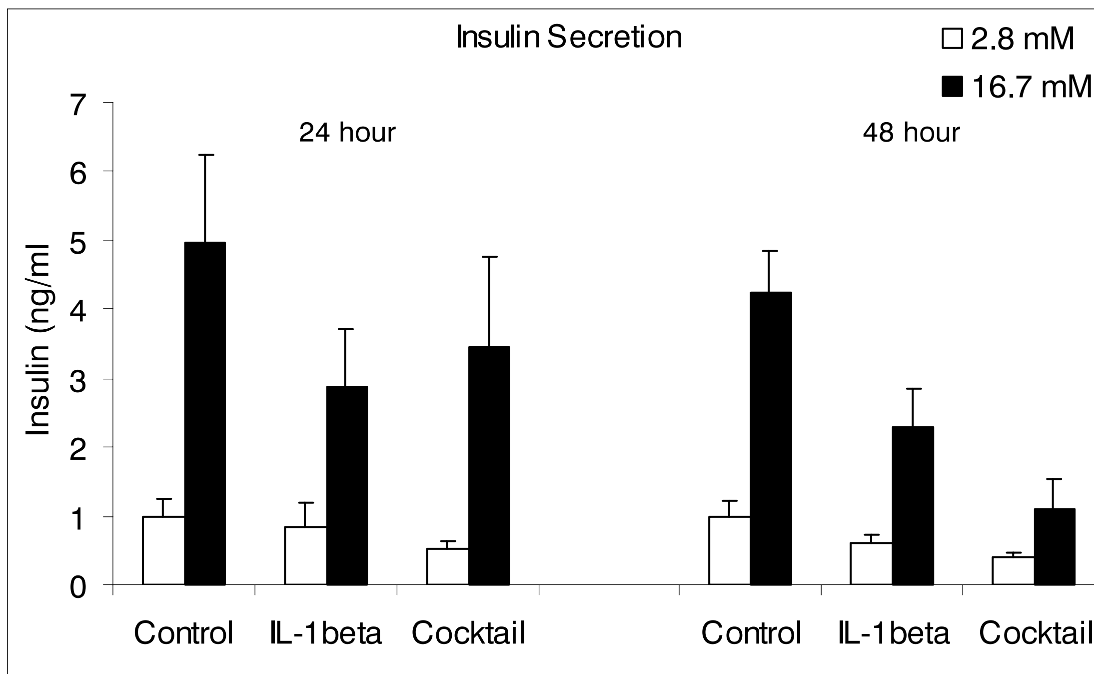


Fig 4.7. MIN6 cells were exposed to either IL1-β (0.5 ng/ml) or a cocktail of IL1-β (0.5 ng/ml), TNF-α (6.25 ng/ml) and IFN-γ (75 ng/ml) for 24 or 48 hours. After the chronic treatment period, the cells were washed and then incubated for 1 hour in media containing either 2.8 or 16.7 mM glucose. Insulin secretion from the MIN6 cells is expressed as the fold change in the level in control cells at 2.8 mM glucose at both 24 and 48 hours. The values are means ± SEM, (n=2).

## 4.2.3 Change in the mRNA level of gene involves in glucose metabolism

To investigative the mechanisms responsible for cytokine-induced β-cell dysfunction, MIN6 cells were treated with cytokines and then RNA was extracted and gene expression analyzed by real-time RT-PCR. Insulin secretion is dependent on glucose transport proteins (GLUT2), glycolytic and mitochondrial enzymes which are essential for metabolism of glucose. Changes in mRNA levels of metabolism enzymes are shown in figure 4.9. Cytokines led to a reduction in mRNA level of GLUT2 of approximately 20 % for IL-1β exposed cells and approximately 40 % for the combined in the cytokine exposed cells compared to control cells.
mRNA levels for the rate limiting electron shuttle enzyme, mitochondrial glycerol-3-phosphate dehydrogenase (mGPDH), tended to decline in the IL-1β exposed cells and a further decrease in the cytokines-cocktail exposed cells.

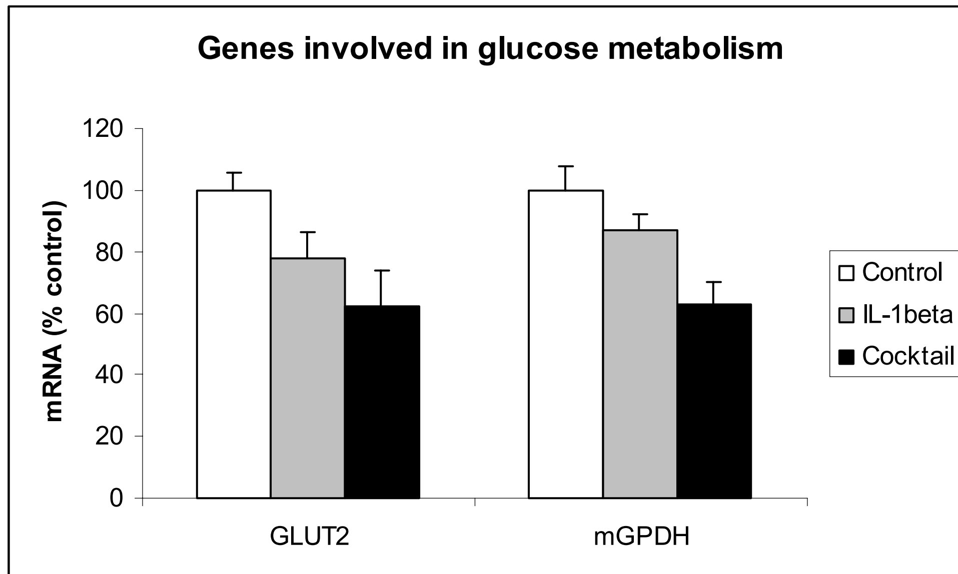


Fig 4.8. mRNA level of the genes involved in the glucose metabolism in MIN6 cells expressed as a percentage of control set at 100 % for each gene. MIN6 cells were exposed to either IL1-β (0.5 ng/ml) or a cocktail of IL1-β (0.5 ng/ml), TNF-α (6.25 ng/ml) and IFN-γ (75 ng/ml) for 24 hours. The values are mean ± SEM, (n=2). GLUT2 = glucose transporter-type 2; mGPDH = mitochondrial glycerol-3-phophate dehydrogenase.

## 4.2.4 Change in the mRNA level of ion channels/pumps

The mRNA level of two central ion channels was measured. Pore-forming subunit of the ATP-sensitive $K^+$ channel (Kir6.2) is the gene coding for the $K^+$-ATP channel. Increases in ATP due to glucose metabolism leads to closure of Kir6.2 and membrane depolarisation, an important step in glucose induced insulin secretion. Thus, increased Kir6.2 could lead to reduced effectiveness of glucose-derived ATP to stimulate insulin secretion. This gene tended to rise in the cytokines exposed cells compared to control cells. In contrast, the calcium transporter-the sarco endoplasmic

$Ca^{2+}$-ATPase 2 (Serca2b) showed reduced mRNA levels in the cytokines exposed cells compared to the control cells. Serca2b is voltage dependent $Ca^{2+}$ channel and is a downstream step of glucose metabolism leading to insulin secretion.

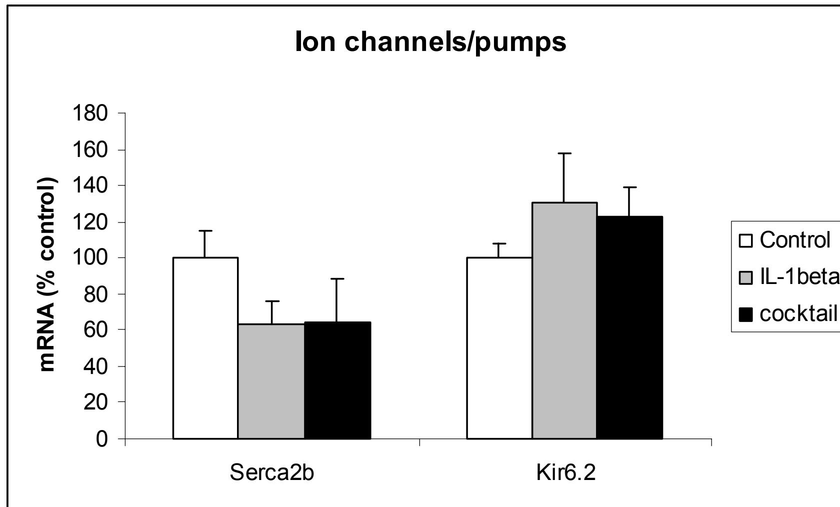


Fig 4.9. mRNA level of ion channels/pumps in the MIN6 cells expressed as a percentage of the control set at 100 % for each gene. MIN6 cells were exposed to either IL1-β (0.5 ng/ml) or a cocktail of IL1-β (0.5 ng/ml), TNF-α (6.25 ng/ml) and IFN-γ (75 ng/ml) for 24 hours. The values are means ± SEM, (n=2). SERCA2b = sarco endoplasmic reticulum $Ca^{2+}$ transport ATPase 2b; Kir6.2 = pore-forming subunit of the ATP-sensitive $K^+$ channel.

### 4.2.5 Change in mRNA level of inducible nitric oxide synthase (iNOS) gene.

To check that the cytokines were acting effectively to induce transcription, we measured mRNA levels of a known target gene, iNOS. In Figure 4.11, mRNA level of iNOS showed a dramatic rise in the IL-1β and combined cytokine-exposed cells.

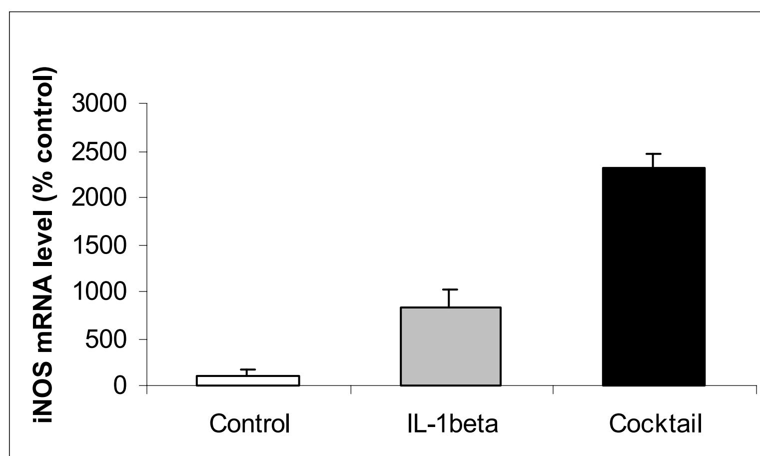


Fig 4.10. mRNA level of inducible nitric oxide synthase (iNOS) gene in the MIN6 cells expressed as a percentage of the control set at 100 %. MIN6 cells were exposed to IL1-β (0.5 ng/ml) or a cocktail of IL1-β (0.5 ng/ml), TNF-α 6.25 ng/ml) and IFN-γ (75 ng/ml) for 24 hours. The values are means ± SEM, (n=2).

### 4.2.6 Change in the mRNA level of Id-1

We also tested Id-1 (inhibitor of differentiation/DNA binding), a negative regulator of basic helix-loop-helix (bHLH) transcription factors normally expressed at low levels in islets and known to be associated with dedifferentiation in other cell types. This has recently been proven in islets of db/db mice and Px rats (unpublished date). Moreover in vitro studies have showed that Id-1 expression is regulated by the glucose in human islets and insulin secreting cell line but not in non β-cell lines. We observed that Id-1 had a 1.5-fold increase in the IL-1β exposed cells and 2-fold increase in the cocktail exposed cells compared to the control cells.

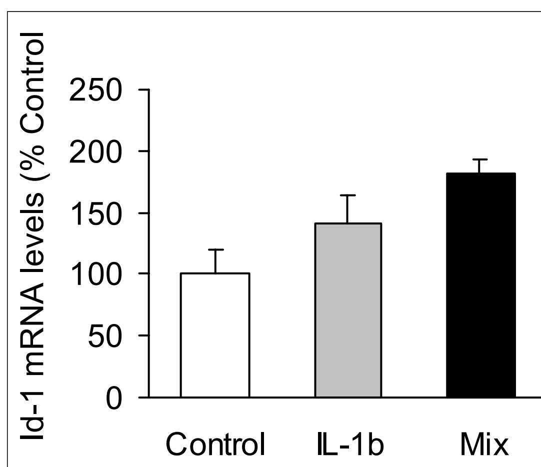


Fig 4.11. mRNA level of the inhibitor of differentiation/DNA binding (Id-1) gene in the MIN6 cells expressed as a percentage of the control set at 100 %. MIN6 cells were exposed to either IL1-β (0.5 ng/ml) or a mix of IL1-β (0.5 ng/ml), TNF-α (6.25 ng/ml) and IFN-γ (75 ng/ml) for 24 hours. The values are means ± SEM, (n=2).

### 4.2.7 Change in MIN6 mRNA level of islet-associated transcription factors

The expression levels of several transcription factors important for pancreas and islet development and the maintenance of β-cell differentiation were altered by cytokine exposure. The pancreatic and duodenal homeobox-1 (PDX-1) has been suggested to control the expression of several glucose metabolism genes. Cytokine exposure led to a decline in PDX-1 mRNA level in MIN6 cells. Mutation of this gene is addressed to the Maturity Onset Diabetes of the Young 4 (MODY4). mRNA level of the MODY1 linked hepatocyte nuclear factor (HNF) -4α transcription factor showed to a decrease in the cytokine exposed cells. Another important transcription factor linked to MODY6, β-cell E-box transactivator 2 (BETA2/NeuroD) mRNA level showed a tendency to decline in the cytokines exposed cells. The mRNA level of paired box gene 6 (Pax6) was observed to have a marked drop in the cytokines exposed cells. A reduction in the NK6 transcription factor related, locus 1 (Nkx6.1) mRNA level was also observed. These findings greatly strengthen the hypothesis that a gradual loss of differentiation underlies the dysfunction of β-cells in diabetes.

The down-regulation of these important islet transcription factors could contribute to the altered expression of genes essential for insulin secretion.

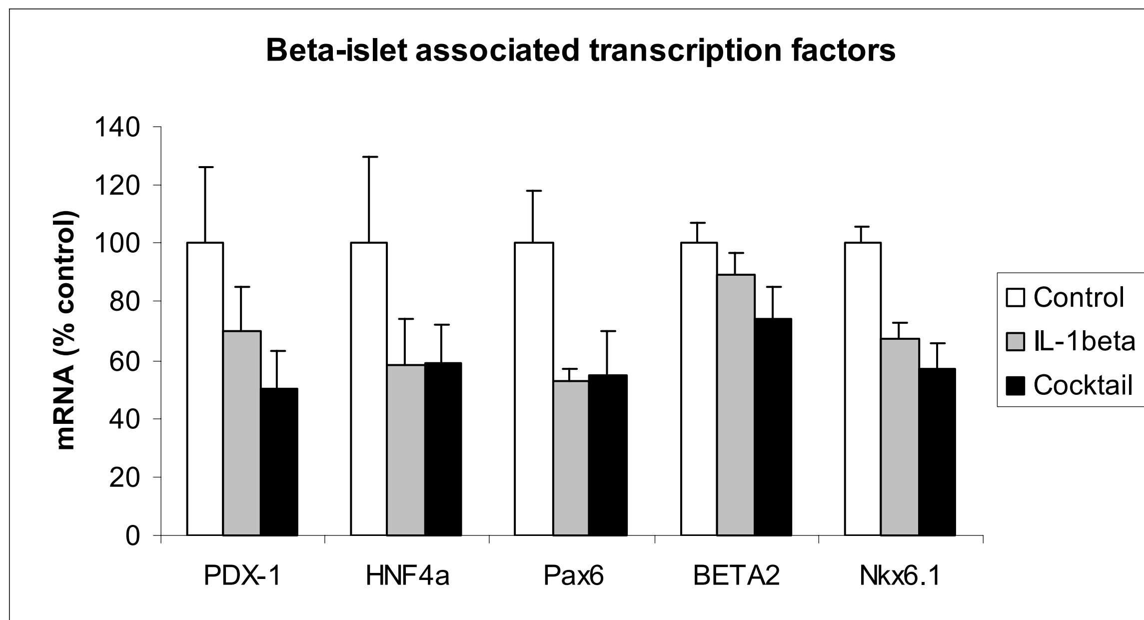


Fig 4.12. mRNA level of the β-islet associated transcription factors in the MIN6 cells expressed as a percentage of the control for each gene set at 100 %. MIN6 cells were exposed to either IL1-β (0.5 ng/ml) or a cocktail of IL1-β (0.5 ng/ml), TNF-α (6.25 ng/ml) and IFN-γ (75 ng/ml) for 24 hours. The values are means ± SEM, (n=2). BETA2 = beta-cell E-box trans-activator 2; HNF4α = hepatocyte nuclear factor 4α; Pax6 = paired box gene 6; PDX-1 = pancreatic and duodenal homeobox-1; Nkx6.1 = NK6 transcription factor related, locus 1.

# 5 DISCUSSION

Many studies have shown that hyperglycaemia and cytokines lead to the loss of glucose stimulated insulin secretion (GSIS) and β-cell apoptosis that characterizes type 2 diabetes (T2D) (Federici, Hribal et al. 2001; Maedler, Spinas et al. 2001; Cardozo, Heimberg et al. 2001; Ling, Van de Casteele et al. 2000; Donath, Gross et al. 1999; Korsgren, Jansson et al. 1990; Gray, Cranston et al. 1989), but the molecular mechanisms responsible are still unclear. In this project, mouse islets and insulin secreting MIN6 cells were used to investigate gene expression changes that correlate with hyperglycaemia- and cytokine- induced β-cell dysfunction and apoptosis. Studies examined the time course changes in gene expression in cells and isolated islets pre-treated with different concentration of glucose and cytokines. The results of these studies have supplied confirmation of several of our initial hypotheses and provided some surprising observations on the detrimental impact of hypoglycaemia, hyperglycaemia and cytokines to increase apoptosis and cause β-cell dysfunction. The findings have expanded our knowledge of the changes in islet and β-cell gene expression, which may underlie the loss of GSIS and β-cell apoptosis that characterizes T2D.

## 5.1 Low glucose levels, rather than high glucose levels induce apoptosis in MIN6 cells

Our studies were initiated on the assumption that prolonged exposure of β-cells to elevated glucose leads to increased rates of β-cell apoptosis. It is important to note that the 25 mM glucose concentration was adopted as the control since this is the normal basal level of glucose in cultured media for MIN6 cells (Busch, Cordery et al. 2002). Interpretation of these experiments must take into consideration that the normal level of glucose exposure to MIN6 cells is higher than the level of glucose exposure in human cells.
Our studies showed that there was only a moderate increase in apoptosis in MIN6 cells cultured in 40 mM compared to 25 mM glucose. While indicating that hyperglycaemia indeed induces a toxic effect in MIN6 cells, the increase in apoptosis was less than expected. In addition, the results clearly show that hypoglycaemia for MIN6 cells (5 mM) led to a dramatically higher percentage of apoptotic cells than hyperglycaemia for MIN6 cells (40 mM). Thus, apoptosis was somehow depressed in these cells exposed to high glucose, raising the possibility that there are mechanisms activated by high glucose that protect the cells from large increases in apoptosis.
As mentioned earlier in the Results section, these results raise two questions: 1) what are the mechanisms responsible for protecting MIN6 cells from overt increases in apoptosis due to high

glucose, and 2) what are the mechanisms responsible for increased apoptosis in cells cultured at low glucose levels? We performed studies to address these important questions as discussed below.

## 5.2 Low glucose levels induce the pro-apoptosis gene CHOP in MIN6 cells

mRNA levels of the gene C/EBP homologous protein (CHOP) were highest in MIN6 cells cultured in low glucose concentration (5 mM). This raises the possibility that the induction of CHOP contributes to the increased apoptosis in β-cells cultured under hypoglycaemic conditions. Intriguingly, other findings in our research group (unpublished data) show a modest increase in CHOP mRNA levels in islets of an animal model of type 2 diabetes (db/db mice) and increased CHOP protein staining in islets of humans with diabetes relative to non-diabetics. These findings suggest that CHOP induction may be a part of the apoptosis stimulus in β-cells. Apoptosis is expected to occur at higher rates in aged db/db mice (Shafrir, Ziv et al. 1999) and in human diabetes (Butler, Janson et al. 2003). In addition, cytokine induced β-cell death appears to act downstream of nitric oxide generation and induction of CHOP (Oyadomari S, Araki E et al. 2002; Oyadomari, Koizumi et al. 2002). CHOP is an apoptotic inducer in the nucleus. The transcriptional induction of this gene is associated with the integrated stress response, which can be activated by diverse stimuli and can be mediated by the transcription factor activating transcription factor 4 (ATF4). In unstressed cells, the mRNA encoding for transcriptional factor ATF4 is basally repressed. However under stress when eukaryotic translation initiation factor 2α (eIF-2α) is phosphorylated, AFT4 is depressed and the encoding protein accumulates in the nucleus. Others eIF-α kinases (not ER stress) can couple AFT4 activation to amino acid starvation and the pathways can also be activated by variety of toxins and conditions that induce oxidative stress, although the kinase(s) implicated in the latter circumstance has not yet been identified. Because these gene expression pathways can integrate signalling by a variety of unrelated stresses, it is referred to it as the integrated stress response (Harding and Ron 2002). Therefore, we can propose a model whereby low glucose activates the integrated stress response (independent of ER stress), which leads to eIF-2α phosphorylation, ATF4 activation, transcriptional up-regulation of CHOP and increased CHOP-induced apoptosis.

Thus, we propose that increased CHOP mRNA levels and apoptosis in cells cultured in low glucose associated are likely linked by the integrated stress response. In contrast, high glucose levels appear to induce endoplasmic reticulum (ER) stress and the protective aspects of the unfolded protein response (UPR) as discussed below.

## 5.3 High glucose levels induce a protective ER stress response in MIN6 cells

Our data support the hypothesis that hyperglycaemia leads to ER stress and the up-regulation of UPR genes in β-cells. Upon disruption of the ER, cells activate a protective response. This involves up-regulation of the genes encoding ER chaperone and degradation proteins to increase protein folding activity, prevent protein aggregation and eliminate misfolded proteins from the ER. In the case of servere ER disruption, ER stress can be a mediator of β-cell apoptosis. In our experiments, ER stress was present in cells treated with high glucose for 48 and 72 h as evidenced by increased mRNA levels for the ER chaperone immunoglobulin heavy chain binding protein (BiP), the protein disulfide isomerase endoplasmic reticulum protein 72 (ERP72) and the ER degradation enchanching α-mannodiase-like protein (EDEM1). Thus, elevated exposure of glucose activates an intracellular signalling pathway from the ER to the nucleus to facilitate these changes. Protein folding in ER is facilitated by ER chaperone proteins such as BiP/GRP178 (Oyadormir, Koizumi et al. 2002) and its up-regulation is characteristic of the UPR. Our results showed that BiP was increased by high glucose in a time-dependent manner. Under stressed condition, BiP dissociates from the transducer proteins inducing their activation. One of these transducer proteins is inositol requiring enzyme 1 (IRE1), which leads to splicing of X-box binding protein 1 (XBP-1) mRNA. The up-regulation of the spliced form XBP-1 in our experiments is another indication of the ER stress response. XBP-1 mRNA is spliced in response to ER stress, resulting in frame shift through which there is a rearrangement to an active form with the original N-terminal DNA binding domain and a new C-terminal transactivation domain. The spliced form of XBP-1 has a higher transcriptional activity in the up-regulation of UPR target genes than unspliced XBP-1 (Oyadormir, Koizumi et al. 2002). Recently, XBP-1 was reported to be a transcription factor specific to the mammalian UPR (Yoshida, Matsiu et al. 2001).

Taken together, our findings of increased UPR genes, BiP, EDEM1, P58, ERP72 and processed XBP-1 all reinforce the notion of an ER stress response due to high glucose. These genes all work downstream the ER transducer proteins IRE1, activating transcription factor 6 (ATF6) and PKR-like endoplasmic reticulum kinase (PERK) (Habener, Kemp et al. 2005), pathways believed to signal the protective response against apoptosis. Consistent with this, mRNA levels of the apoptosis inducer, CHOP were not different in MIN6 cells cultured in 40 mM glucose compared to control-treated cells (25 mM) over the time course studied here. We believe that initially, activation of the UPR may protect β-cell undergoing ER stress. Study of isolated islets from db/db mice with stable β-cell mass gave similar change in these genes expression (unpublished data from Laybutt DR et al. 2006). Interestingly, a study of MIN6 cells treated with toxic concentrations of the fatty acid (FA)

palmitate (saturated FA), but not non-toxic oleate (unsaturated FA) induced CHOP and apoptosis, and to a lesser extent the protective genes of the UPR. We do not discount the likelihood that with more prolonged exposure to high glucose-induced ER stress, UPR signalling may switch to activate cell death.

The progressive up-regulation of ER stress pathways by elevated glucose concentrations occurs in a specific and regulated manner in MIN6 cells. Our results show no meaningful changes in the mRNA level of NK6 transcription factor related, locus 1 (Nkx6.1) gene in MIN6 cells exposed to different glucose levels, indicating the treatments are well-tolerated by the cells throughout the course of these experiments. In Nkx6.1 mutants β-cell numbers are selectively reduced, while other islets cell types develop normally demonstrating a key role of Nkx6.1 in β-cell development (Henseleit, Nelson et al. 2005).
IRS-2 is a key gene in maintaining optimal β-cell mass to compensate for peripheral insulin resistance (Whiters, Burks et al. 1999; Whiters, Gutierez et al. 1998). IRS-2-knockout mice exhibit insulin resistance in both peripheral and liver, and this combined with decrease β-cell mass leads to development of diabetes (Kido, Burks et al. 2000; Whiters, Gutierez et al. 1998).
We found no defect in IRS-2 in high glucose-treated MIN6 cells; conversely we saw an increase with hyperglycaemia indicative of healthy, growing cells.

## 5.4 Time-course change in ER stress genes mRNA level in DBA/2 and C57BL/6 mouse islets

Studies by Zraika and co-workers (Zraika Aston-Mourney et al. 2006) showed that high glucose culture caused impairment in GSIS in DBA/2 islets, a strain of mice with a genetic predisposition to failure. C57BL/6 mouse islets were used as control in this study. Since there are recognized differences in the susceptibility of these mouse strains to β-cell dysfunction, we tested the regulation of ER stress genes in isolated islets from DBA/2 and C57BL/6 mouse. The results showed an increase mRNA level of the UPR genes (BiP, EDEM1, P58 and processed XBP-1) in mouse islets cultured in high glucose. This reinforced our finding in MIN6 cells that hyperglycaemia activates a protective ER stress response. It was also observed a decrease in the mRNA level in CHOP and AFT4 in low glucose compared to high glucose similar to our finding in the MIN6 cells. As stated above, these two genes are believed to be a part of the integrated stress response downstream of eIF2α phosphorylation. Thus, the studies in mouse islets nicely complement our findings in the MIN6 cells.

However, our study did not show any meaningful differences in hyperglycaemia-induced ER stress gene changes between the DBA/2 and the C57BL/6 mice. Thus, it is unlikely that changes in the ER stress response contribute to the different susceptibility of these mouse strains to β-cell dysfunction.

## 5.5 Loss of GSIS in cytokines exposed MIN6 cells

It was mentioned earlier that hyperglycaemia activates the immune system causing inflammation and the release of the inflammatory factors or cytokines, which are believed to cause β-cell dysfunction and cell death (Cnop, Welsh et al. 2005). We have, in this project investigated changes in insulin secretion in MIN6 cells cultured in cytokines. We observed the predictable increases in insulin secretion in response to acute (1 h) glucose stimulation in control cells. Adding cytokines to the media somehow disrupts this secretion. We also showed that loss in GSIS is time dependent. This finding raises the question of what are the mechanisms responsible for cytokine-induced loss of GSIS? The mechanisms are not known yet, but may involve several triggering enhancing activity of the nuclear transcription factor NF-κB, which plays a critical role in mediating inflammatory responses (Cardozo, Heimberg et al. 2001; Grey, Arvelo et al. 2001).

## 5.6 Changes in mRNA level of islets associated transcription factors due to cytokines

We tested if cytokines can lead to β-cell dedifferentiation in MIN6 cells. The results of gene expression studies involved in β-cell differentiation were strikingly similar with studies in the db/db mouse model of diabetes (Kjørholt, Åkerfeldt et al. 2005). mRNA level of several transcription factors important for maintenance of β-cell differentiation (PDX-1, HNF-4, Pax6, BETA2 and Nkx6.1) were reduced with cytokine exposure. Down-regulation of these important islets transcription factors are also linked to the altered expression of genes essential for insulin secretion. Recent studies of the biology of pancreas development have shed new light on the function of these transcription factors. Much of this understanding has been obtained by analyses of the phenotypes of mice in which the expression of these genes has been disrupted (knockout mice). Remarkably, almost without exception, disruption of these genes has resulted in phenotypes of impaired development of the pancreas and consequent diabetes subtype linked to Maturity Onset Diabetes of the Young (MODY) (Habener, Kemp et al. 2005).

## 5.7 The role of changes in enzymes and ion-channel expression in the loss of GSIS

The loss of GSIS has been revealed in T2D and early T1D and many diabetic animal models (Leahy, Bonner-Weir et al. 1992) and the db/db mice show a similar defect in GSIS (Kjørholt, Åkerfeldt et al. 2005; Thorens, Wu et al. 1992; Berglund, Frankel et al. 1978). Like other diabetic animal models the glucose transporter-type 2 (GLUT2) mRNA was decreased in the MIN6 cells exposed to cytokines. A similar study in rat pancreatic β-cell cultured in cytokines also observed a decrease in the GLUT2 gene expression (Cardozo, Heimberg et al. 2001). The down regulation of the GLUT2 is associated with reduced β-cell response to glucose. However, this alone can not fully explain the defect. We tested another metabolism gene, mitochondrial glycerol-3-phophate dehydrogenase (mGPDH), and it also showed a decreased mRNA expression level. Down-regulation of these metabolic enzymes suggested a decrease in glucose metabolism and consequently disruption in the pathways leading to production of ATP, which is essential for glucose stimulated insulin secretion. Studies of animals of T2D (Jonas, Sharma et al. 1999; Tokuyama, Sturlis et al. 1995; Thorens, Weir et al. 1990) and in panceatectomized (Px) rats (Jonas, Sharma et al. 1999) found similar changes in the expression of metabolism enzymes as we found here with cytokines.

The mRNA levels of ion channels important for stimulation of insulin secretion, the ATP sensitive $K^+$ channel, Kir6.2 was increased, while the $Ca^{2+}$-ATPase, SERCA2b was decreased in these studies. This finding of Kir6.2 gene expression is distinct from other studies where the proximal portion of the $K^+$ channel Kir6.2 subunit was down-regulated (Laybutt, Glandt et al. 2003; Tokuyama, Sturlis et al. 1995). In contrast our results of the endoplasmic reticulum $Ca^{2+}$-ATPase gene SERCA2b had a similar down-regulated change in gene expression as in a study of diabetic islets of Goto-Kakzaki (GK) rats (Vardadi, Molnar et al. 1996). This suggests that we have to take the test environment into consideration. We predict that increased in Kir6.2 would lead to reduced effectiveness of glucose derived ATP to stimulate insulin secretion. Decreases in SERCA2b may lead to altered calcium handling by the cell and thus to decrease in insulin secretion. These data support that hypothesis that cytokines lead to widespread changes in gene expression consistent with a loss of β-cell differentiation.

## 5.8 The role of the cell cycle gene Id-1 in β-cell dedifferentiation

Several studies performed in diabetic animal models, suggests that β-cell have a remarkable capacity to increase their mass to maintain normoglycaemia, even in presence of obesity and insulin resistance (Butler, Jansson et al. 2003; Weir, Laybutt et al. 2001; Bonner-Weir S. 2000). Falling insulin production in diabetes correlates with a progressive decrease in β-cell mass.
We tested the inhibitor of differentiation/DNA binding transcription factor, Id-1, that has recently been proven to be normally expressed at low levels in islets and known to be associated with in other cell types with dedifferentiation. Our findings are consistent with diabetic models of mice and Px rats where this cell cycle gene was up-regulated (Laybutt, Sharma et al. 2002; Kaneto, Sharma et al. 2002; Scheuner, Song et al. 2001; Ruderman, Saha et al. 1998). The up-regulating of this cell cycle gene Id-1 is suggested to be associated with β-cell dedifferentiation, reduced glucose induced ATP synthesis and a loss of GSIS. In addition, it has also been demonstrated that glucose regulates the expression of Id-1 in human islets and in insulin-secreting cell lines but not in liver and other non β-cell lines (Webb, Akbar et al. 2001; Wice, Bernal-Mizrachi et al. 2001). Our findings in this study are consistent with the hypothesis that increased Id-1 leads to the loss of β-cell phenotype and impaired GSIS in cytokines-treated β-cells.

# 6 CONCLUSION AND FURTHER PERSPECTIVES

## 6.1 Conclusion

In conclusion, the findings of these studies support our hypothesis that hyperglycaemia and cytokines lead to ER stress and β-cell dedifferentiation of pancreatic β-cells. We demonstrated that hyperglycaemia leads to activation of the unfolded protein response associated with endoplasmic reticulum (ER) stress. Several key genes (ERP72, BiP, EDEM1, P58, XBP-1) that are associated specifically with ER stress response were up-regulated in both isolated mouse islets and MIN6 cells treated with high levels of glucose for 48-72 h. Under these conditions, the cells were largely protected from apoptosis. In contrast, hypoglycaemia leads to an integrated stress response with the up-regulation of CHOP and increased apoptosis occurring in the absence of ER stress.

Other studies showed that chronic exposure of MIN6 cells to cytokines leads to decreased expression of genes (PDX-1, HNF4α, Pax6, BETA 2 and Nkx6.1) that are thought to be involved in the specialized function and maturation of the β-cell phenotype. This was associated with reduced expression of metabolic enzymes and ion channel (GLUT2, mGPDH and Serca2b) and increased mRNA levels for a gene capable of inhibiting differentiation, Id-1. Theoretically, these changes in gene expression could be responsible for cytokine-induced β-cell dysfunction. Finally more experiments needs to be performed to confirm that these results are statistically significant.

## 6.2 Future perspectives

Future works could focus on the gene inhibitor of differentiation/DNA binding (Id-1) in MIN6 cells, and examine its role in β-cell differentiation, function and survival. Experiments should be carried out to determine whether altered expression of the Id-1 plays a necessary role in the β-cell dysfunction and cell death induced by high glucose and cytokines.
Another interesting line of work would be to investigate the influence of reactive oxygen species (ROS) to the change in gene expression. This will be determined by treating the cultures with the antioxidant drug N-acetyl-cysteine cells (NAC). If DNA binding activity of NF-κB is increased by long-term culture in high glucose and cytokines, its role in the changes in β-cell phenotype could be investigated by treating the cells with the nonsteroidal anti-inflammatory drug sodium salicylat, a

NF-κB inhibitor, and testing whether inhibition of NF-κB activation prevents high glucose/cytokine induced dedifferentiation and the loss of GSIS.

# 7 REFERENCES


Benezra, R., Davis, R.L. et al. (1990). *The protein Id: a negative regulator of helix-loop-helix DNA binding proteins*. Cell 61:49-59.

Berglund, O., Frankel, B.J. et al. (1978). *Development of the insulin secretory defect in genetically diabetic db/db mouse.* Acta Endocrinol (Copenh). 87(3):543-51.

Bertolotti, A., Zhang, Y. et al. (2000). *Dynamic interaction of BIP and ER-stress transducers in the unfolded- protein response*. Nat Cell Biol 2:326-332.

Biarnes, M., Montolio, M. et al. (2002). *Beta cell death and mass in syngeneically transplanted islets exposed to shorts and long term hyperglycemia*. Diabetes 51(1):66-72.

Bishop, A.E. and Polak, J.M. (1997). *The anatomy, organization and ultra structure of the islets of langerhans*. In textbook of diabetes, edited by Pickup J.C. and William G., Blackwell Sciences Ltds, Oxford (1997) 6.1-6.16.

Bonner-Weir, S.(2000). *Islets growth and development in the adult*. J Mol Endocrinol 24(3):297-302.

Busch A.K, Cordery D et al. (2002). *Expression profiling of palmitate- and oleate-regulated genes provides novel insights into the effects of chronic lipid exposure on pancreatic beta-cell function.* Diabetes 51:977-987

Butler, A.E., Janson, J. et al. (2003). *β-cell deficit and increased β-cell apoptosis in human with type 2 diabetes.* Diabetes 52:102-110.

Cardozo, A.K., Heimberg, H. et al. (2001). *A comprehensive analysis of cytokineinduced and nuclear factor κB-dependent genes primary rat pancreatic β-cells.* Journal Biol Chem 276(52)48879-48886.

Cardozo, A.K., Ortis, F. et al. (2005). *Cyokines downregulate the sarcoplasmic reticulum pump $Ca^{2+}$-ATPase 2b and deplete endoplasmic reticulum $Ca^{2+}$, leading to induction of endoplasmic reticulum stress in pancreatic β-cells*. Diabetes 54:452-61.

Cnop, M., Welsh, N. et al. (2005). *Mechanisms of peancreatic β-cell death in type 1 and type 2 diabetes. Many differences, few similarities*. Diabetes 54(2):97-107.

Cordle, S.R., Henderson, E. et al. (1991). *Pancreatic β-cell type specific transcription of the insulin gene is mediatd by basic helix-loop-helix DNA binding proteins*. Mol Cell Biol 11:1734-1738.

Deeney, J.T., Prentki, M. et al (2000). *Metabolic control of β-cell function.* Semin Cell Dev Biol 11:267-275.

Donath, M.Y., Ehses, E.K. et al. (2005). *Mechanisms of β-cell death in type 2 diabetes*. Diabetes 54:108-113.

Donath, M.Y., Gross, D.J. et al. (1999). *Hyperglycemia -induced β-cell apoptosis in pancreatic islets of Psammomys obesus during development of diabetes*. Diabetes 48:738-44.

Evans, J.L., Goldfine, I.D. et al. (2002). *Oxidative stress and stress –activated signalling pathways: a unifying hypothesis of type 2 diabetes*. Endocrine Rev 23:599-622.

Federici, M., Hribal, M. et al. (2001). *High glucose causes apoptosis in cultured human pancreatic islets of langerhans: a potential role for regulation of specific Bcl family genes toward an apoptotic cell death program.* Diabetes 50:1290-1301.

Gray, D.W., Cranston, D. et al. (1989). *The effect of hyperglycemia on pancreatic islets transplanted into rats beneath the kidney capsule*. Diabetologica 32:663-667.

Grey, S.T., Arvelo, M.B. et al. (1999). *A20 inhibits cytokine-induced apoptosis and nuclear factor kappaB-dependent gene activation in islets*. J Exp Med 190:1135-1146.

Guz, Y., Montminy, M.R. et al. (1995*). Expression of murine STF-1, a putative insulin gene exretion and endocrine progenitor during ontogeny*. Development 121(1):11-18.

Habener, J.F., Kemp, D.M. et al. (2005). *Transcriptional regulation in pancreatic development.* Endocrinology 146(3):1025-1036.

Halban, P.A., Kahn, S.E. et al (2001). *Gene and cell-replacement theraphy in the treatment of type 1 diabetes: how high must the standards be set?* Diabetes 50:2181-2191.

Harding, H.P. and Ron, D. (2002). *Endoplasmic reticulum stress and the development of diabetes.* Diabetes 51:445-461.

Harding, H.P., Zeng H. et al. (2001). *Diabetes mellitus and exocrine pancreatic dysfunction in perk-/- mice reveals a role for transplantational control in secretory cell survival*. Mol Cell 7:1153-1161.

Henseleit, K.D., Nelson, S.B. et al. (2005). *Nkx6 transcription factor activity is required for alpha- and beta-cell development in the pancreas*. Development 132(13):3139-49.

Huang, H., Tsai, M. et al. (2000). *Transcription factors involved in pancreatic islet development*. J Biomed Sci 7:27-34.

Jensen, J., Serup, P. et al. (1996). *mRNA profiling of the tumors reveals Nkx6.1 as a β-cell specific homeodomain transcription factor.* J Biol Chem 271:18749-18758.

Jonas, J.C., Sharma A. et al. (1999). *Chronic hyperglycemia triggers loss of pancreatic β-cell differentiation in an animal model of diabetes*. J Biol Chem 274:14112-21.

Jonsson, J., Carlsson, L. et al. (1994). *Insulin promoter factor 1 is required for pancreas development mice*. Nature 371(6498):606-9.

Kaneto, H., Sharma K. at al (2002). *Induction of c-Myc expression suppresses insulin gene*

*transcription by inhibiting NeuroD/BETA2-mediated transcriptional activation*. J Biol Chem 277:12998-3006.

Kharroubi, I., Ladriere, L. et al. (2004*). Free fatty acids and cytokines induce pancreatic β-cell apoptosis by different mechanisms: role of nuclear factor-kappaB and endoplasmic reticulum stress*. Endocrinology 145:5087-96.

Kido, Y., Burks, D.J. et al. (2000). *Tissue-specific insulin resistance in mice with mutation in the insulin receptor IRS-1 and IRS-2*. J Clin Inv 105(2):199-205.

Kjørholt, C. (2003). *Beta-cell dysfunction in an animal model of diabetes and obesity, the C57BI/ksJ db/db mouse.* Masterthesis.School of pharmacy, University of Oslo, Norway.

Kjørholt, C., Åkerfeldt, MC. et al. (2005). *Chronic hyperglycemia, independent of plasma lipid level, is sufficient for the loss of beta-cell differentiation and secretory function in the db/db muose model of diabetes*. Diabetologica 54(9):2755-63.

Komatsu, M., Yajima, H. et al. (1999). *Augmentation of $Ca^{+}$ stimulated insulin release by glucose and long-chain fatty acids in rat pancreatic islets; free fatty acids mimic ATP-sensitive $K^{+}$ channel-independent insulinotropic action of glucose*. Diabetes 48(8):1543-9.

Korsgren, O., Jansson, L. et al. (1990). *Hyperglycemia-induced β-cell toxicity. The fate of pancreatic islets transplanted into diabetic mice is dependent on their genetic background*. J Clin Invest 86:2161-2168.

Laybutt, D.R., Glandt, M. et al. (2003). *Critical reduction in beta-cell mass results in two distinct outcomes over time. Adaptation with impaired glucose tolerance or decompensated diabetes*. J Biol Chem 278(5):2997-3005.

Laybutt, D.R., Sharma, A. et al. (2002). *Genetic regulation of metabolic pathways in β-cells disruption by hyperglycemia*. J Biol Chem 277(13):10912-10921.

Laybutt, D.R., Weir, G.C. et al. (2002). *Genetic regulation of metabolic pathways in β-cells disrupted by hyperglycemia*. J Biol Chem 277:10912-21.

Leahy, J.L., Bonner-Weir, S. et al. (1992). *β-cell dysfunction induced by chronic hyperglycemia*. Diabetes Care 15(3):442-455.

Ling, Z., Van de Casteele, M. et al. (2000*). Interleukin-1β-induced alteration in a β-cell phenotype can reduce cellular sensitivity to conductions that cause necrosis but not to cytokin -induced apoptosis.* Diabetes 49:340-345.

Lingohr, M.K., buettaer, .R et al. (2002). *Pancreatic β-cell growth and survival: a role in obesity-linked type 2 diabetic?* Trends Mol Med 8(8):375-384.

Maechler, P. and Wollheim, C.B. (1999). *Mitochondrial glutamate acts as a messenger in glucose-induced insulin exocytosis*. Nature 402(6762):685-9.

Maedler, K., Spinas, G.A. et al. (2001). *Glucose induces β-cell apoptosis via up-regulation of the*

*Fas receptor in human islets*. Diabetes 50:1683-90.

Marshark, S., Leibowitz, G. et al. (1999*). Impaired β-cell function induced by chronic exposure of cultured human pancreatic islets to high glucose*. Diabetes 48:1230-1236.

Naya, F.J., Huang H.P. Et al. (1997). *Diabetes, defective pancreatic morphogenesis, and abnormal enteroendocrine differentiation in BETA2/neuroD- deficient mice*. Genes Dev 11(18):2323-34.

Naya, F.J., Stellrecht, C.M. et al. (1995). *Tissue-specific regulation of the insulin gene by a novel basic helix-loop-helix transcriptional factor*. Genes Dev 9(8):1009-19.

Nelson, D.L. and Cox M.M. (2000). In *Lehninger: Principles of Biochemistry*. Third ed., Worth Publisher, USA.

Newgard, C.B. and Matchinsky F. (2000). Substrate control of insulin release. A chapter for "Handbook of physiology" Jefferson L., Cherrington A., Editors.

Norton J.D (2000). *Id helix-loop-helix proteins in cell growth, differentiation and tumorgenesis*. J Cell Sci 113:3897-907.

Ogawa, Y., Noma, Y. et al. (1995). *Loss of glucose-induced insulin secretion and GLUT2 expression in transplanted beta-cells*. Diabetes 44:75-79.

Orthega-Cammarille, C., Guzman-Grenfell, A.M. et al. (2006). *Hyperglycemia induces apoptosis and p53 mobilization to mitochondria in RINm5F cells*. Mol and Cell Bio Chem 281(1-2):163-171.

Oyadomari, S., Koizumi, A. et al. (2002). *Targeted disruption of the Chop gene delays endoplasmic reticulum stress-mediated diabetes.* J Clin Invest 109:525-532.

Oyadormari, S., Araki, E. et al. (2002). *Endoplasmatic reticulum stress-metdiated apoptosis in pancreatic beta cell.* Apoptosis 7:335-345.

Pick, A., Clark, J. et al. (1998). *Role of pancreatic beta-cell mass compensation for insulin resistance and beta-cell defects in the male Zucker diabetic fatty rat*. Diabetes 47:358-364.

Pretkin, M. (1996). *New insights into pancreatic beta cell metabolism signaling in insulin secretion*. Eur J Endocrinol 134(3):274-86.

Ruderman, N.B., Saha A.K. et al. (1998). *Malonyl CoA as a metabolic switch and a regulator if insulin sensitivity.* Adv Exp Med Biol 441:263.70.

Sakai, K., Matsumoto, K. et al. (2003*). Mitochondrial reactive oxygen species reduce insulin secretion by pancreatic beta-cells*. Biochem biophys Res Commun 300(1):216-22.

Sander, M. and German M.S. (1997). *The beta cell transcription factors and development of the pancreas.* J Mol Med 75(5):327-40.

Scheuner, D., Song B. et al. (2001). *Transcriptional control is required for the unfolded protein response and in vivo glucose homeostasis*. Mol Cell 7:1165-76.

Servitja, J.M. and Ferrer, J. (2004). *Transcriptional networks controlling pancreatic development and beta-cell function*. Diabetologia 47:597-613.

Shafrir, E., Ziv E. et al. (1999). *Nutritionally induced insulin resistance and receptor defect leading to beta-cell failure animal models*. Ann NY Acad Sci 892:223-46.

Shimabukuro, M., Zhou, Y.T. et al. (1998). *Fatty acid-induced beta-cell apoptosis: a link between obesity and diabetes*. Proc Natl Acad Sci USA 95:2498-2502.

Sikder, H.A., Devlin, M.K. et al. (2003*). Id protein in cell growth and tumorigenesis*. Cancer Cell 3:525-30.

Stoffers D.A., Thomas M.K. et al. (1997*). Homeodomain protein IDX-1: a master regulator of pancreatic development and insulin gene expression*. Trends Endo Metab 8:145-151.

Stoffers, D.A., Zinkin, N.T. et al. (1997). *Pancreatic agenesis attributeable to a single nucleotide deletion in the human IPF1 gene coding sequence.* Nat Genet 15(1):106-10.

Sussel, L., Kalamaras, J. et al. (1998). *Mice lacking the homeodomain transcription factor Nkx2.2 have diabetes due to arrested differentiation of pancreatic β-cells*. Development 125:2213-2221.

Thorens, B., Weir, G.C. et al. (1990*). Reduced expression of the liver/beta-cell glucose transporter isoform in glucose-sensitive pancreatic beta-cells of diabetic rats*. Proc Natl Acad Sci USA. 87(17):6492-6.

Thorens, B., Wu Y.J. et al. (1992). *The loss of GLUT2 expression by glucose-unresponsive beta cells of db/db mice is reversible and is induced by the diabetic enviroment*. J Clin Invest 90(1):77-85.

Tokuyama, Y., Sturlis, J. et al. (1995*). Evolution of beta-cell dysfunction in the male Zucker diabetic fatty rats*. Diabetes 44(12):1447-57.

Varadi, A., Lebel L. et al. (1999). *Sequence variants of the sarco(endo)plasmic reticulum Ca(2+) transport ATPase 3 gene (SERCA3) in caucasian tupe II diabetic patients (UK Prospective Diabetes Study 48).* Diabetologica 42(10):1240-3.

Waeber, G., Thompson, N. et al. (1996). *Transcriptional activation of the Glut 2 gene by the IDF-1/STF-1/IDX-1 homeobox factor.* Mol Endocr 10:1327-1334.

Wang, W., Lawson, B. et al. (1996). *Signal from the stressed endoplasmic reticulum induce C/EBD-homologeus protein (CHP/GADD153).* Mol Cell Biol 16:4273-4280.

Watada, H., Katjimoto, et al. (1996). *PdX-1 induce insulin and glucokinase gene expression in α-TC1 clone 6 cells in the presence of betacellulin.* Diabetes 45:1826-31.

Webb, G.C., Akbar, M.S. et al. (2001). *Expression profiling of pancreatic β-cells. Glucose regulation of secretory and metabolic pathway genes*. Diabetes 50(1):135-136.

Weir, G., Laybutt, D.R. et al. (2001). *β-cell adaptation and decompensation during the progression*

*of diabetes*. Diabetes 5(1):154-159.

Weir, G.C., Bonner-weir, S. et al. (2004). *Five stage of evolving β-cell dysfunction during progression to diabetes*. Diabtetes 53(3):16-21.

Wice, B.M., Bernal-Mizrachi, E. et al. (2001). *Glucose and other insulin secretagogues induce, rather than inhibit, expression of Id-1 and Id-3 in pancreatic islets beta cell*. Diabetologica 44:453-463

Withler, D.J., Burks, D.J. et al. (1999). *IRS2 coordinates IGF-2 receptor mediated β-cell development and peripheral insulin signaling*. Nat Genet 23(1):32-40.

Withler, D.J., Gutierrez, J.S. et al.(1998). *Disruption of IRS2 cause type 2 diabetes in mice*. Nature 391:900-904.

Yokota, Y. and Moris, S. et al. (2002). *Role of Id family proteins in growth control*. J Cell Physiol 190:21-28.

Yoshida, H., Matsui, T. et al. (2001). *XBP-1 mRNA is induced by ATF6 and spliced by IRE1 in response to ER stress produce a highly active transcription factor*. Cell 107(7):881-91.

Zhang, K., Kaufman, R.J. et al. (2004). *Signalling the unfolded protein response from the endoplasmic reticulum*. J Biol Chem 279:25935-8.

Zimmet, P., Alberti, K.G. Et al. (2001). *Global and societal implications of the diabetes epidemic*. Nature 414:782-787.

Zraika, S., Aston-Mourney K. et al. (2006). *The influence of genetic background on the induction of oxidative stress and impaired insulin secretion in mouse islets*. Diabetologica 49(6):1254-63.