The impact of glucotoxicity on pancreatic β-cell function

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To my parents

"To do successful research, you don't need to know everything; you just need to know of one thing that isn't known."

Arthur Schawlow

Contents

Abbreviations

AC	Adenylate cyclase
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanonsine monophosphate
cAMP-GEFII	cAMP-regulated gaunaine nucleotide exchange factor
ChREBP	Carbohydrate responsive element-binding protein
DAG	Diacylglycerol
FFA	Free fatty acid
GRP	Gastrin releasing peptide
GLP1	Glucagon-like peptide 1
GIP	Glucose dependent insulinotropic peptide
GLUT4	Glucose transporter 4
GSIS	Glucose-stimulated insulin secretion
GTP	Guanosine triphosphate
GK rat	Goto-Kakizaki rat
GPR40	G protein coupled receptor 40
HDL	High-density lipoprotein
HSP60	Heat shock protein 60
IP3	Inositol triphosphate
IL-1β	Interleukin- 1 beta
IMM	Inner mitochondrial membrane
INS1	Insulinoma 1 cell line
IBMX	3-isobutyl-1-methylxanthine
LDL	Low-density lipoprotein
NADPH	Nicotinamide-adenine dinucleptide phosphate
NO	Nitric oxide
NOS	Nitric oxide synthase
nNOS	neuronal NOS
INOS	inducible NOS
eNOS	endothelial NOS
OMM	Outer mitochondrial membranes

PP	Pancreatic polypeptide
PACAP	Pituitary adenylate cyclase –activating polypeptide
PKC	Protein Kinase C
PKA	Protein kinase A
PDE	Phosphodiesterase enzyme
PhA	Phosphatidic acid
ROS	Reactive oxygen species
TG	Triglyceride
TNF –α	Tumor necrosis factor-alpha
T2D	Type 2 diabetes
TXNIP	Thioredoxin interacting protein
VIP	Vasocative intestinal peptide
VDAC	Voltage-Dependent Anion Channels
ZDF rat	Zucker Diabetic Fatty Rat

List of Original Papers Included in the Thesis

1. **Muhammed SJ**, Lundquist I, Salehi A. Pancreatic β-cell dysfunction, expression of iNOS and the effect of phosphodiesterase inhibitors in human pancreatic islets of type 2 diabetes. Diabetes Obes Metab. 2012 Jun 11.
2. Sandra Meidute Abaraviciene, **Sarheed J. Muhammed**, Stefan Amisten, Björn Olde, Ingmar Lundquist and Albert Salehi. GPR40 protein levels are crucial to the differential regulation of stimulated hormone secretion in pancreatic islets. Lessons from spontaneous obesity-prone and non-obese type 2 diabetes in rats. *Submitted.*
3. Ahmed M, **Muhammed SJ**, Kessler B, Salehi A. Mitochondrial proteome analysis reveals altered expression of voltage dependent anion channels in pancreatic β-cells exposed to high glucose. Islets**.** 2010 Sep-Oct; 2(5):283-92.
4. **Sarheed J Muhammed**, Arvind Soni, Enming Zhang, Meftun Ahmed, Vladimir V. Sharoyko, Erik Renström, Leif Groop, Claes B. Wollheim, Albert Salehi. Altered expression of voltage dependent anion channels are involved in pancreatic β-cells dysfunction. *Manuscript*

Publications not included in this thesis

1. Taneera J, Jin Z, Jin Y, **Muhammed SJ**, Zhang E, Lang S, Salehi A, Korsgren O, Renström E, Groop L, Birnir B. γ-Aminobutyric acid (GABA) signalling in human pancreatic islets is altered in type 2 diabetes. Diabetologia (2012) 55:1985–1994
2. Paloma Alonso-Magdalena, Ana B Ropero, Marta García-Arévalo, Sergi Soriano, Iván Quesada, **Sarheed J. Muhammed**, Albert Salehi, Jan-Ake Gustafsson and Angel Nadal. Antidiabetic actions of an ERβ selective agonist. *Manuscript*
3. Salehi A, Gunnerud U, **Muhammed SJ**, Ostman E, Holst JJ, Björck I, Rorsman P. The insulinogenic effect of whey protein is partially mediated by a direct effect of amino acids and GIP on beta-cells. Nutr Metab (Lond). 2012 May 30; 9(1):48.
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5. Soriano S, Alonso-Magdalena P, García-Arévalo M, Novials A, **Muhammed SJ**, Salehi A, Gustafsson JA, Quesada I, Nadal A. Rapid insulinotropic action of low doses of bisphenol-A on mouse and human islets of Langerhans: role of estrogen receptor β. PLoS One. 2012; 7 (2):e31109

Introduction

Diabetes mellitus is a group of metabolic diseases that share the common property of chronic hyperglycemia [1]. The incidence of diabetes is increasing worldwide, it was estimated by International Diabetes Federation (IDF) in 2001 that the worldwide prevalence of type 2 diabetes (T2D) amounts ~ 177 million of adult population. This number is expected to rise to an estimated 300 million by the year of 2025 [2]. T2D is the most common form of diabetes and results from a complex interaction between genes and environment. A genetic element clearly underlies β-cell dysfunction and insufficient β-cell mass; however, a number of modifiable factors are also linked to β-cell deterioration, most notably chronic hyperglycemia and elevated free fatty acid (FFA) levels [3]. In type 2 diabetes, chronic hyperglycemia has long been felt to have negative consequences on β-cell function. Chronic elevation of blood glucose concentration impairs β-cell function and insulin sensitivity, a phenomenon referred to as glucotoxicity [4, 5]. In addition to defected glucose balance diabetic patients also have increased levels of plasma FFA. Indeed, it has been suggested that chronic exposure to elevated level of FFAs don't hurt the cell unless glucose concentrations are elevated as well [6].

Hyperglycemia can cause long-term complications and may cause cardiovascular diseases, retinopathy, nephropathy and neuropathy [7]. The cost of diabetes to national and individual economies is wasteful, largely related to complications of disease.

Despite the intensive efforts to investigate the pathogenesis of diabetes, the triggering factors and underlying mechanisms behind the development of diabetes remain elusive. Therefore, further understanding of development of the condition is highly motivated. This thesis deals with effect of glucotoxic condition on pancreatic β-cell function and functional mechanisms in type 2 diabetes mellitus.

The Endocrine Pancreas

The pancreas consists of two functionally different groups of cells. Cells of the exocrine pancreas produce and secrete digestive enzymes and fluids into the upper part of small intestine. The endocrine pancreas which an anatomically constitutes small portion of pancreas (2-3 % of the total mass), consists of numerous discrete clusters of cells, known as islets of Langerhans, which are located throughout the pancreas. The islets of Langerhans are considered as functional units of endocrine pancreas, which play a crucial role in regulating fuel storage and glucose homeostasis. Islets composed of several cell types: insulin secreting β-cells, glucagon secreting α-cells, somatostatin secreting δ-cells and pancreatic polypeptide (PP) - secreting cells. The α, δ, and PP cells are located near the periphery while the β-cells typically occupy the most central part of islets and comprising 70%-80 of the endocrine cells [8].

The islets have a very rich blood supply which accounts for about 10% of the total pancreatic blood flow during resting conditions, the volume being increased when glucose levels are high [9]. Afferent blood vessels penetrate nearly to the center of islets before branching out and returning to surface of islet. The efferent capillaries merge into collecting venules and drain into the venous system [10]. The vascularization of endocrine part of pancreas differs from that of exocrine pancreas in that they are thinner walled and wider and have more fenestrations, enabling an extensive exchange of molecules [11].

The endocrine pancreas is also provided with complex neural supply, which includes parasympathetic, sympathetic and sensory nerves. Most of the nerve fibers enter the pancreas along the arteries [12]. There are four different neurotransmitters localized to islet parasympathetic nerves (acetylcholine, VIP, PACAP and GRP), which are released by activation of vagal nerve and stimulate insulin and glucagon secretion [13] While the sympathetic nerves inhibit insulin secretion and stimulate glucagon secretion, although, β-adrenergic activation exerts stimulatory action [14].

Beta Cells Produce Insulin

Beta cells are the most numerous cell types of the islets. They are generally wide and contain secretory granules. Beta cells secrete insulin, proinsulin, C-peptide, and amylin. Insulin, proinsulin and C-peptide are only made in pancreatic β-cells. Insulin is a polypeptide composed of two chains (A and B) held together by disulfide bonds and it is synthesized from proinsulin precursor molecules by the action of proteolytic enzymes known as prohormone convertases (PC1 and PC2), as well as the exoprotease carboxypeptidase E. Insulin is stored in secretory vesicles that are released into the capillaries surrounding the β-cells upon metabolic demand. It has been shown that insulin is secreted in pulsatile manner [15]. Increased actions of insulin have been reported when hormone delivered in pulsatile manner versus a constant manner [16]. Glucose is the most potent stimulus of insulin but also increased plasma concentration of some amino acids, especially arginine, leucine, and lysine; GLP1and GIP released from the gut following a meal; and parasympathetic via the vagus nerve acts as stimulator [17, 18]. Physiologically insulin decreases blood glucose levels by suppressing hepatic glucose production and by stimulating peripheral glucose uptake by fat and muscle tissues. Insulin promotes glucose uptake by muscle and fat tissues via stimulation GLUT4 from intracellular to plasma membrane. On the other hand, the β-cells are provided with GLUT2 transporter that permits glucose entry into the β-cells in direct proportion to extracellular levels [19].it has been shown an inhibitory effect of insulin on glucagon and somatostatin secretion whereas the effect of insulin on its own secretion is still controversial [20].

Alpha Cells Produce Glucagon

Alpha cells comprise most of the remaining cells of islets. They are responsible for synthesizing and secreting glucagon. Glucagon is a peptide hormone which synthesized as proglucagon and proteolytically processed to generate glucagon within α-cells of pancreatic islets. The major effect of glucagon is to stimulate an increase in blood concentration of glucose. Glucagon stimulates the breakdown of glycogen stored in the liver and activates hepatic gluconeogenesis thereby increasing glucose production. Glucagon also has a great effect of enhancing lipolysis in adipose tissue, which could be considered as an added means of conserving blood glucose level by providing fatty acid fuel into most cells. Glucagon produces the above mentioned effects through binding to specific receptor in the cell

membrane. These receptors are linked to a G-protein exerting effects on cAMP levels [21]. Glucagon secretion inhibited by both insulin and somatostatin [22].

Delta Cells Produce Somatostatin

Delta cells are sites of production of somatostatin in the pancreas. These cells are typically located in the periphery of the islet often between β-cells and surrounding mantle of α-cells. In addition, somatostatin is also found in the central and peripheral nervous system, and numerous tissues including gastrointestinal tract [23, 24]. Somatostatin is regarded as inhibitory hormone which inhibits the release of insulin glucagon and pancreatic polypeptide [25].

PP Cells Produce Pancreatic Polypeptide

PP-cells are the least abundant pancreatic islet cells, representing only 1% of the total cell population. Most of the PP-cells are found in the head of pancreas and little is found in body and tail [26]. In human, the plasma concentrations of PP increase significantly after meal [27]. It is secreted in a biphasic manner in proportion to food intake, and plasma levels of the peptide remain elevated for up to 6 h [28]. Adrenergic stimulation due to hypoglycemia or exercise leads to an increase in plasma PP levels [29, 30]. The physiologic importance of PP-cells is yet to be determined.

There are many other hormones beside insulin, glucagon, somatostatin and pancreatic polypeptide, are produced in the islets cells for which the functional role is not fully assumed. Islets amyloid polypeptide (IAPP or amylin) is synthesized in pancreatic β-cells [31, 32] and normally cosecreted with insulin [33]. Ghrelin was recently found to be expressed in pancreatic islets [34].It has been documented that peptide YY (PYY) producing cells located in the islets of Langerhans and they are observed either alone or co-localized with glucagon or PP [35].

Diabetes mellitus

Diabetes mellitus is a group of metabolic diseases associated with hyperglycemia and glucose intolerance, due to inadequate insulin supply [36].
On the basis of etiology and clinical presentation the disorder has been classified into four major types [37].

1- *Type 1 diabetes mellitus* (β-Cell destruction, usually leading to absolute insulin deficiency)
   a- Immune -mediated
   b- Idiopathic

2- *Type 2 diabetes mellitus* (Ranging from predominantly insulin resistance with relative insulin deficiency to a predominantly an insulin secretory defect with insulin resistance)

3- *Other specific types* (include more uncommon types of diabetes like maturity-onset diabetes of young (MODY) which is a subtype of non-insulin-dependent diabetes mellitus and caused by genetic defect)

4- *Gestational diabetes mellitus* (GDM) (any degree of glucose intolerance with onset during pregnancy)

Type 2 diabetes mellitus

T2D is the most common form of diabetes mellitus, accounts for more than %90 of all diabetes cases [38]. T2D is a metabolic disorder that is primarily characterized by pancreatic β-cell failure (β-cells do not produce enough insulin) and insulin resistance (reduced response to insulin) in target tissues, which leads to hyperglycemia (Fig 1). The mechanisms behind the insulin resistance are not fully understood, but it is closely connected to obesity, elevated FFA, sedentary lifestyle and overproduction of stress hormones [39]. Although obesity itself causes insulin resistance [40] not all obese

subjects develop diabetes since the insulin-producing β-cells adapt by increasing insulin secretion to maintain normal plasma glucose levels [41].

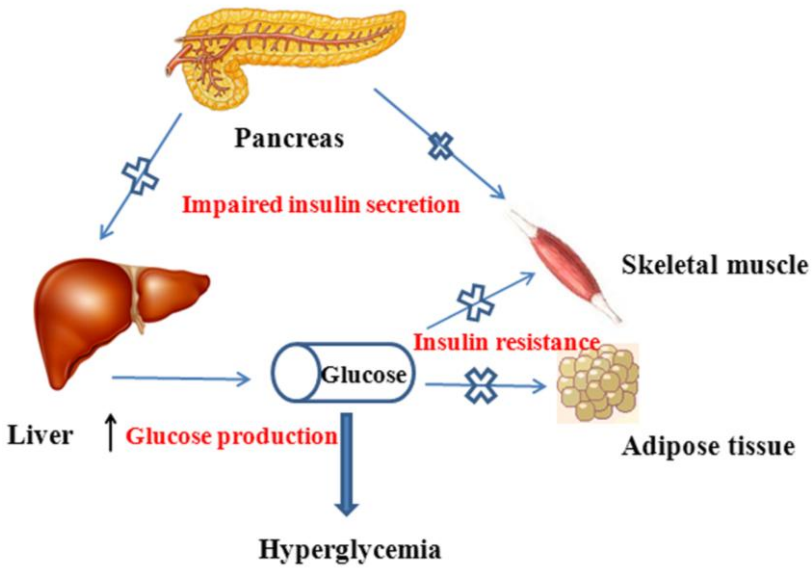

Figure 1. **Pathophysiology of hyperglycemia in type 2 diabetes**
T2D is caused by three major metabolic defects: increased hepatic glucose output, impaired pancreatic insulin secretion and peripheral insulin resistance which resulting in hyperglycemia.

Thus, a progressive loss of β-cell function has a vital role in the pathogenesis of T2D. In reality, the impaired β-cell function often comes before manifestation of insulin resistance [42]. The exact mechanisms behind β-cell dysfunction remain unclear but glucose uptake abnormality, failure of β-cell to respond to glucose, reduced β-cell mass and defected exocytotic machinery are considered as the main mechanisms [43-46]. However, there is now strong evidence that the failure of insulin secretion is causally related to development of T2D [47].

Characteristic features of insulin release

Insulin is secreted uniquely from pancreatic β-cells and has a major role in maintaining energy homeostasis. The release of insulin stimulated by intracellular signals obtained from metabolism of nutrients, of which glucose provides the primary stimulus [48], However, several other molecules like FFA, amino acids, and keto acids also affect the stimulus-secretion coupling [49, 50]. Glucose-stimulated insulin secretion normally is biphasic, an initial first transient phase followed by a slowly developing second phase of insulin release. Elevation of the glucose concentration is followed by a rapid and transient stimulation of insulin which rapidly drops to basal levels and thereafter increases in oscillatory manner and remains for several hours if the β-cell is continuously exposed to glucose [51, 52]. In T2D, first-phase of insulin release is almost lost, and second-phase is strongly reduced [53]. Reduction of first-phase insulin secretion takes place early in the course of T2D, as it has been documented in subjects with impaired glucose tolerance [54], as well as normoglycaemic first-degree relatives of patients of T2D [55]. In addition to nutrients, there are a number of hormones and neuromodulators, which stimulate insulin secretion, including GLP1 that increases cAMP levels through specific G-protein-coupled receptors [56]. Another pathway acts through binding of cholinergic agents to muscarinic receptors, which stimulates the production of intracellular second messenger molecules like (IP3 and DAG) and thus increases intracellular calcium concentrations and promotes protein kinase C activity [57-59].

Glucose-stimulated insulin secretion

In pancreatic β-cells, glucose metabolism is crucial for GSIS. Normal glucose stimulated insulin secretion requires glucose sensing, where oxidative mitochondrial metabolism plays a vital role [60, 61]. When blood glucose is high, glucose enters the pancreatic β-cell via specific glucose transporters (GLUT-2 in rodent and GLUT-1 in human) and then rapidly is phosphorylated by glucokinase [62, 63]. Glucose-stimulated insulin secretion from pancreatic beta cells is regulated by two main signaling pathways (Fig 2).

1- The K_{ATP} channel dependent pathway which is also called the triggering pathway. Glucose enters the β-cell through specific GLUT-transporters and then is metabolized in the cytoplasm and mitochondria, which results in an increase in the ATP/ADP ratio and this leads to closure of the (K_{ATP}) channels. Depolarization of plasma membrane occurs upon closure of (K_{ATP}) channels which resulting in activation of voltage-dependent L-type Ca^{2+} channels, followed by an influx and rise of cytosolic Ca^{2+} concentration which finally triggers insulin release through the exocytosis of insulin–containing granules [64, 65]. This pathway is mainly involved in the first-phase of insulin release but it has been documented that it is acting also, to some degree during second-phase of insulin secretion [66, 67].

2- The K_{ATP} channel independent pathway which is also referred to as the amplifying pathway of glucose-induced insulin secretion. This pathway augments the glucose-stimulated secretory response in the presence of increased Ca^{2+} concentrations [51, 68, 69]. The amplifying pathway is involved in second phase of insulin secretion [51, 70]. Although the mechanism behind the amplifying pathway has not yet been fully understood but several factors have been suggested that they are operating through this pathway in amplifying insulin secretion, like cAMP, glutamate, FFA, NADPH, ATP and GTP [71-75].

In healthy adults, there is a precise balance between insulin secretion from pancreatic β-cells and insulin action on sensitive peripheral tissues to keep the plasma glucose concentration within normal range (4-7 mM). If the tissues, primarily liver, adipose tissue and skeletal muscle, become less responsive to the action of insulin, then the β-cells have to compensate by secreting enough insulin to maintain normoglycemia. The pancreatic β-cells can often manage this increased demand for insulin by increasing their number or capacity, resulting in hyperinsulinemia. However, if reduced glucose-lowering effects of insulin progresses further, the pancreatic β-cell hypersecretion of insulin may fail to compensate for insulin resistance and this may eventually leads to β-cell exhaustion and ultimately β-cell death in susceptible individuals [36]. So elevated blood glucose levels for prolonged

period of time would have adverse effects on different tissue functions, a process called glucotoxicity. Several mechanisms for the glucotoxic damage have been proposed of which decreased transcription of insulin gene by a reduced binding of important β-cell transcription factors [76, 77]. Chronic hyperglycemia has been associated with increased production of reactive oxygen species (ROS), which may cause mitochondrial damage [60]. It has been shown also that prolonged exposure to elevated glucose concentration motivates an increase of glucagon release which in turn increases hepatic glucose output [78]. Moreover, it has been suggested that chronic hyperlipidemia may don't harm the pancreatic β-cells as long as it is not accompanied by hyperglycemia [79].

Free Fatty Acid receptor (GPR40)

Under normal circumstances free fatty acids (FFA) is regarded as important physiological fuels for pancreatic β-cells. FFAs augment insulin release acutely but prolonged exposure to FFAs has lipotoxic effect on the pancreas [80]. There are two hypotheses regarding FFA involvement in the augmentation of GSIS. First hypothesis is that FFA taken up by β-cells with subsequent intracellular formation of long-chain acyl-CoA (LC-CoA) in the cytoplasm is the cause [81] and that (LC-CoA) may directly affect the secretory mechanisms [82] or indirectly through the formation of complex lipids like TG, PhA, and DAG, stimulates insulin release [83]. Second hypothesis is based on the identification of GPR40 as FFA receptor. GPR40 is activated by medium- to long-chain FFAs in the micromolar range [84]. GPR40 is highly expressed in pancreatic β-cells and the K and L cells of the small and large intestine respectively [85, 86]. It has been documented that GPR40 is also expressed in α-cells of pancreatic islets [87]. GPR40 plays an important role in mediating the majority of effects of FFAs on pancreatic β-cells [88]. GPR40 is coupled to $G_{\alpha q}$ protein with a subsequent increase in cytosolic Ca^{2+} concentration as well as activation of PKC [89]. The possible role of GPR40 in insulin secretion is suggested by observations that GPR40-deficient mice (GPR40−/−) did not show enhancement of GSIS after acute FFA exposure [90]. Moreover the GPR40−/− mice were protected against the deleterious effects of a high fat

diet [90]. This suggests GPR40 involvement in both acute and chronic effects of FFAs on insulin release.

cAMP- potentiated Insulin Secretion

Glucagon and incretins like glucagon-like peptide-1 (GLP1) and glucose dependent insulinotropic peptide (GIP) enhance glucose stimulated insulin secretion by activating second messenger pathways (Fig 2) [91-94]. This is accomplished by binding to specific receptors on the β-cell surface, followed by activation of the G-protein-coupled adenylate cyclase (AC) which causes an increase in intracellular cAMP. This leads to cAMP-dependent activation of second messenger pathways like protein kinase A (PKA).

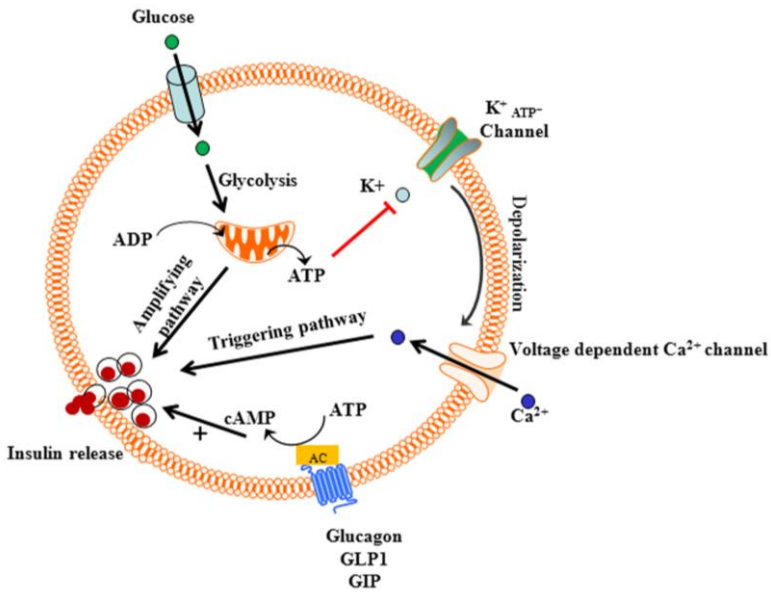

Figure 2. **Schematic overview of pathways involved in insulin secretion**
The triggering pathway begins with the uptake and metabolism of glucose generating ATP which closes the K^+_{ATP}-channels. This leads to membrane depolarization, which opens voltage dependent Ca^{2+} channels and Ca^{2+} flux into the cell and triggers the release of insulin granules. The amplifying pathway enhances the rate of Ca^{2+}-induced insulin release. Peptide hormones, like glucagon, GLP1, and GIP potentiate GSIS through the increased cAMP formation by AC.

This PKA dependent pathway stimulates insulin release by increasing influx of Ca^{2+} through voltage-dependent Ca^{+2} channels [95, 96]. However, cAMP has also been found to stimulate insulin secretion by PKA-independent pathway that acts through cAMP-regulated guanine nucleotide exchange factor (cAMP-GEFII) also known as exchange protein associated with cAMP (Epac) [97]. This pathway has been suggested to affect exocytotic process [98]. Moreover, it has been shown that cAMP, in addition to these effects, also improves β-cell survival through PKA-dependent pathway [99]. The concentration of cAMP in pancreatic β-cell is preserved by a balance between its formation via the activity of adenylel cyclases (ACs) and its destruction by cyclic nucleotide phosphodiesterases (PDEs) [100].

Cyclic nucleotide phosphodiesterases (PDEs)

PDEs consist of a family of enzymes the function of which is the degradation of cAMP and cGMP [101, 102]. There are now 11PDE families (PDE1-PDE11) detected with at least 100 PDE enzymes [101, 102]. Most families are composed of several subfamilies, in some cases encoded by different genes. The PDE genes generate several protein products by alternative splicing or transcription. All PDEs share 3 functional domains catalytic domain, a regulatory N-terminus and C-terminus [103-105]. The catalytic domain is homologous between different PDEs and located in C-terminal part of protein. The N-terminal domain shows variance between PDE families and contains regions that determine intracellular localization [106]. The PDE families are characterized by the differences in their primary structures, selectivity for cAMP and cGMP substrates, sensitivity to effectors and inhibitors, tissue distributions, regulation, expression, cellular functions and susceptibility to pharmacological inhibitions [107-109]. The cAMP-specific enzymes comprise PDE4, -7, and -8. The cGMP-specific PDEs are PDE5, -6, and -9, whereas PDE1, -2, -3, -10, -11 use both cAMP and cGMP [108]. The role of specific PDE isoforms in different physiological processes has been understood by the discovery of selective PDE inhibitors [110]. Availability of family-specific PDE 1, 2, 3, 4 and 5 inhibitors made easier understanding the role of these individual PDE in different tissues. For instance, PDE3 inhibitors have been shown to relax vascular and airway smooth muscle, prevent platelet aggregation [111] and induce lipolysis [112]. PDE4

inhibitors were shown to be highly effective at suppressing inflammation [113] whereas PDE5 inhibitors play a role in regulating smooth muscle tension [114]. Sildenafil, a selective PDE5 inhibitor, is used as a therapeutic agent in treating erectile dysfunction in man [115]. In relation to the work in this thesis (Paper I), PDE1/5, PDE3, and PDE4 inhibitors have been used to clarify their role in the regulation of insulin secretion in human pancreatic islets.

Role of β-cell PDEs in glucose-stimulated insulin secretion

Several studies have been published with the aim to characterize the expression of PDEs in β-cells and to identify their role in regulation of insulin release. Thus, by using selective PDE inhibitors, PDE1, PDE3 and PDE4 have been identified in β-cells [116-120]. The relative contribution of these PDEs has not been fully elucidated in glucose-induced insulin secretion. Inhibition of PDE1 in mouse islets but not in human and rat islets has been reported to potentiate glucose-induced insulin release [116, 119, 121]. Data from cell lines indicates that inhibition of PDE1 in β-TC3 [119] seems to augment glucose-induced insulin secretion but not in BRIN-BD11 cells [120]. Moreover PDE1/PDE5 inhibitor has been used in BRIN-BD11 cells and islets, and was ineffective in glucose- stimulated insulin release [116, 120]. So the role of PDE1 in regulation of glucose-stimulated insulin release varies.

With regard to PDE3, there are 2 subfamilies of PDE3 family (PDE3A and PDE3B) [122, 123]. These enzymes are transcripted from two different genes in human located on chromosome 12 and 11, respectively [122-124]. PDE3s exhibit a high affinity for both cAMP and cGMP; however the V_{max} for cAMP is about 4-11folds greater than cGMP [125, 126]. They are characterized also by their sensitivity to several selective inhibitors like cilostamide, milrinone and imazodan [127]. Another characteristic of PDEs is that they have tissue-specific expression and subcellular localization [128, 129]. PDE3A is mainly expressed in heart, vascular smooth muscle, oocytes and platelets [130]. PDE3B is predominantly found in adipocytes, hepatocytes, spermatocytes and pancreatic β-cells [131]. So generally, PDE3B expression is high in cells that are crucial for the regulation of energy homeostasis while PDE3A expression is high in cells of cardiovascular system [132]. There is strong document for a role of PDE3B in the regulation of cAMP-mediated insulin secretion [116, 121]. It

has been shown that inhibition of PDE3 by using different PDE3 selective inhibitors potentiates glucose-induced insulin secretion form rat and human islets [121, 133]. Moreover, data from *in vivo* experiments have indicated that PDE3 inhibitors act as insulin secretagogues both in rodents and human [117, 134, 135]. The role of PDE4 in the regulation of glucose stimulated insulin secretion differs depending on the cell type used. Inhibition of PDE4 in both β-TC3 and BRIN-BD11cells results in enhancement of glucose-stimulated insulin release [119, 120] whereas in islets inhibition of PDE4 not to significantly potentiate insulin secretion in response to glucose [116, 121].

Influence of Nitric Oxide on Endocrine Pancreas

Nitric oxide is a gaseous signaling molecule that arranges different physiological and pathophysiological actions in the human body. these include vascular smooth muscle relaxation, neurotransmission, inhibition of platelet aggregation, exocrine and endocrine function [136]. It plays also important role in immune system since macrophages activated by cytokines, can produce large amount of NO which functions as antimicrobial and tumoricidal molecule both in vivo and in vitro [137]. NO is highly lipophilic free radical. It is extremely reactive and has a very short half- life [138]. NO is produced in many tissues by three distinct isoforms of NO synthase (NOS), neuronal NOS-1 (nNOS), inducible NOS-2 (iNOS) and endothelial NOS-3 (eNOS). Whereas iNOS is inducible, eNOS and nNOS are constitutively expressed [139]. The NOS enzymes are bidomain proteins. NOS activity requires binding of reductase domain of one NOS monomer with oxygenase domain of its partner for proper NO production. The NOS dimer uses nicotinamide adenine dinucleotide phosphate oxidase (NADH/NADPH), tetrahydrobiopterin (BH 4) cofactor and oxygen (O 2) to convert its substrate, L -arginine, to L –citrulline and NO [140]. NO has a variety of effects depending on its relative concentration and the surrounding milieu in which NO produced. NO protects the cell at pico- to nanomolar concentrations. At higher concentrations, NO and its derivatives become cytotoxic. There are both direct effects of NO that mediated by NO itself and indirect effect of NO that are mediated by reactive nitrogen species produced by reaction of NO with superoxide anion or with oxygen.

There are several mechanisms by which NO have been demonstrated to affect the biology of the living cells. *Firstly* NO reacts easily with transition metals like iron, copper, and zinc. When NO interact with prosthetic iron groups such as heme, it can form complexes that activate or inactivate target enzymes [141]. *Secondly* NO can induce the formation of S-nitrosothiols from cysteine residues in a reaction called S-nitrosylation . S-nitrosylation is a mechanism for dynamic, post-translational regulation of many important classes of protein. S-nitrosylation inactivates many proteins [142]. *Thirdly* NO reacts very quickly with superoxide anion (O_2^-), resulting in formation of peroxynitrite ($ONOO^-$), which is even more reactive than NO itself [142]. Excessive NO formation leads to nitrated proteins, inhibition of mitochondrial respiration, DNA damage, apoptosis and necrotic cell death, resulting in tissue injury [143, 144]. The first mechanism is considered as direct effects of NO while the two latter mechanisms are referred as indirect effects of NO.

Constitutively expressed nNOS and eNOS are Ca^{2+} - calmodulin dependent. nNOS is expressed in the central and peripheral nervous system, skeletal muscle and pancreatic islets [145-147]. In the brain, nNOS acts as neuromodulator while in peripheral nervous system acts as neurotransmitter, participating in different physiological processes. The functions attributed to nNOS needs rapid and localized production of NO and timely termination of synthesis. Thus nNOS regulation is mediated through the obligatory binding of calmodulin, which occurs only in response to transient increases in intracellular Ca^{2+}. nNOS generates NO in short bursts following activating signals. The physiological concentrations of NO produced by this isoform is in the picomoalr range [148]. eNOS is predominantly present in the endothelium of blood vessels [149, 150]. This isoform is involved in maintaining blood pressure and inhibition of platelet aggregation. The activity of eNOS requires binding of calmodulin, and this binding occurs in response to transient increases in intracellular Ca^{2+} [151]. The eNOS produces NO in pulsatile manner. Physiological concentrations of eNOS-derived NO are in the picomolar range [148]. iNOS is Ca^{2+}-calmodulin independent. It binds calmodulin tightly even at very low concentration of Ca^{2+} [151, 152]. The activity of iNOS isn't responsive to Ca^{2+} oscillations and thus this isoform produces NO in large amount (nmol) for prolonged periods of time [148]. iNOS is expressed in macrophages, hepatocytes, keratinocytes, endothelium, respiratory epithelia, inflammatory cells, and β-cells [137, 153]. The iNOS gene is under

transcriptional control of cytokines and inflammatory mediator [141, 154]. Overproduction of NO in the wrong place leads to tissue destruction, inflammation and induction of cell apoptosis [155, 156]. Considerable evidences, including RT-PCR, immunofluorescence, and western blot analyses have shown expression of nNOS and eNOS normally and iNOS under pathophysiological conditions in the pancreatic islets [157-159]. nNOS-derived NO is increasingly regarded as an important messenger molecule in different kinds of cellular processes including insulin release [158-160]. However, the role of nNOS which generates NO in pulsatile manner in a small amount, in insulin release is not fully understood, but from studies in mouse and rat pancreatic islets, it appears that pancreatic β-cell nNOS exerts inhibitory effect on insulin secretion [158, 159]. With regard to iNOS, previous studies have suggested that iNOS can only be expressed in pathophysiological conditions in response to inflammatory cytokines like IL-1β, TNF-α, and lipopolysaccharide [155, 161, 162]. Excessive NO production from iNOS in cytokine-activated β-cells, is cytotoxic and has been implicated in β-cell destruction in type I insulin-dependent diabetes mellitus (IDDM) [155, 161, 162]. However non-cytokine induction of iNOS, in the presence of high glucose in pancreatic islets, has been documented [159, 163, 164]. This of great importance since overproduction of NO by iNOS is reportedly harmful to β-cells [155, 161, 162]. The mechanism of glucose-induced iNOS expression is poorly understood. It has been suggested that NADPH generated by pentose shunt in glucose metabolism, is an important factor in IL-1β induction of iNOS [165, 166]. It is well established that chronic hyperglycemia is detrimental to pancreatic β-cells and has been implicated in the pathogenesis of T2D as a process called glucotoxicity [167]. In this context, it is credible that iNOS derived NO might be a contributing factor in this process [159].

Voltage-Dependent Anion Channels (VDAC) and Pancreatic Beta cell function

Mitochondria play an essential function in glucose-stimulated insulin secretion in pancreatic β-cells. In normal pancreatic islets, insulin secretion is regulated by glucose metabolism and mitochondria are a major site where important metabolites that regulate insulin secretion are released as we outlined before. The important functional mitochondria for normal GSIS

are demonstrated by alterations in different mitochondrial protein expression levels, which has been associated with impaired GSIS [168].

The Voltage-Dependent Anion Channel (VDAC) was first discovered in 1976 and has been extensively studied in recent years. VDACs, also known as mitochondrial porin are the most abundant proteins which are located on outer mitochondrial membrane [169]. They form pores that coordinate cross-talk between mitochondria and cytoplasm by transporting small anions (*e.g.* phosphate, ATP and ADP), cations (*e.g.* Ca^{2+}, Na^{+} and K^{+}) and metabolites [170]. Mitochondrial membrane potential controls the fluxes of the ions and metabolites by changing the channel's selectivity and permeability. At low voltages (10 mV), the VDAC channel is stable in a long-lived open state (up to 2 h). At high positive or negative potentials (>40 mV), VDAC shows multiple sub-states with different ionic selectivities and permeabilities [171, 172]. The VDAC channel shifts to closed states when the trans-membrane voltage exceeds 20–30 mV. In mammals, three VDAC isoforms; VDAC1, VDAC2 and VDAC3, have been identified with VDAC1 and VDAC2 ubiquitously expressed in tissues (including pancreas, skeletal muscle, heart, liver, brain, placenta, lung and kidney) while VDAC3 has a more restricted distribution [173]. The approximate molecular weights of all mammalian VDAC proteins range between 30–35-kDa [174]. They share also the same protein structure, featuring a 19-stranded β-barrel and N-terminal α-helical region located inside the pore, in different species [175, 176]. It has been documented that VDAC is also expressed in the plasma membrane which indicates that VDAC has more novel functions [177]. In addition to transporting small molecules between the mitochondria and the cytosol, VDAC has been implicated in mitochondria-mediated apoptosis. There are several models proposed for its involvement in cell apoptosis. In one, VDAC is considered as a part of the permeability transition pore (PTP) which composes the adenine nucleotide translocase (ANT) in the IMM, cyclophilin D in the mitochondrial matrix, creatine kinase in the intermembrane space, and hexokinase (HK) associated with VDAC in the cytosol (Fig 3). It has been suggested that the PTP is activated by pro-apoptotic signals like reactive oxygen species in the mitochondria to release cytochrome c via VDAC. Second model, VDAC interacts with Bcl-2 family proteins (Bax and tBid), which leads to structural modifications and enlarging that allowing cytochrome c to exit mitochondria through OMM. Third model, VDAC closes, causing mitochondrial intermembrane space swelling and membrane

rupture, followed by the release of cytochrome c [173]. The diversity in function is attributable to different isoform of protein. For instance, VDAC1 has been implicated in apoptosis through interaction with proteins in the Bcl-2 family [178] while VDAC2 has been associated with anti-apoptotoic activity by inhibiting Bak-mediated apoptosis [179]. Overexpression of VDAC1 in different kind of cells, in mouse, rat, and human, has been associated with apoptosis [180]. Although VDAC has been extensively studied, there is only scant information about VDAC role in the pathophysiology of diabetes. Papers III and IV in this thesis, dissect the mechanism how VDAC involved in altered secretory function of pancreatic β-cell under glucotoxic conditions.

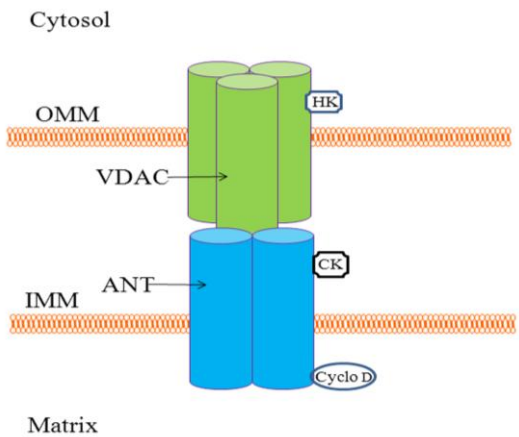

Figure 3. **Schematic structure of the mitochondrial permeability transition pore (mPTP).** It consists of the voltage-dependent anion channel (VDAC) on the outer mitochondrial membranes (OMM), the adenine nucleotide translocator (ANT) on the inner mitochondrial membranes (IMM), cyclophilin D (Cyclo D) in the matrix. Other proteins like hexokinase (HK) and creatine kinase (CK), might also be associated with the mPTP.

Aims of the Thesis

The overall aim of the present study was to delineate the mechanisms involved in glucotoxicity in pancreatic islets and insulin-producing β-cell line. The specific aims were to identify such mechanisms by measuring:

- iNOS expression and its relationship to the cAMP system in islets from human diabetic and non-diabetic donors.
- GPR40 expression in relation to palmitate-induced hormone release in pancreatic islets of obese and lean diabetic animal models.
- Complex changes in protein patterns of mitochondria isolated from INS-1E cells cultured at normal and elevated glucose concentrations.
- Altered VDAC1 and VDAC2 expression in islets isolated from diabetic and non-diabetic donors as well as in INS-1 832/13 and isolated islets from non-diabetic donors under glucotoxic conditions.

Material and Methods

Animals (paper II)

Male Zucker diabetic fatty fa/fa rats and their controls (Charles River Laboratories International, Inc. Kisslegg, Germany) as well as male GK rats (Möllegård, Ry, Denmark) with their Wistar controls weighing 150-175 g (6-8 weeks) were used in our experiments. All animals were given a standard pellet diet (B&K, Sollentuna, Sweden) and tap water *ad libitum* throughout the experiments. The experimental procedure were approved by the Ethical Committee for Animal Research at University of Lund; Sweden and were in accordance with the international standard recommended by NIH.

Rat pancreatic islets (paper II)

Preparation of isolated pancreatic islets was performed by retrograde injection of an ice-cold collagenase solution (3-5 ml) via the bile-pancreatic duct as previously described [181]. Thereafter pancreas was dissected out and carefully separated from surrounding tissue and then placed in tubes (20 ml) and subsequently in water bath (30 cycles /minute) at 37°C for 17 min. the pancreatic islets were separated from acinar tissue by vigorous shaking in ice-cold Hank's solution for several minutes. After sedimentation for about 10-15 min, the islets were handpicked under stereomicroscope at room temperature and then subjected to different experimental procedures.

Human pancreatic islets (papers I and IV)

Isolated human pancreatic islets from non-diabetic (HbA1c: 5.7±0.14) and type 2 diabetic subjects (HbA1c: 6.4±0.25) were provided by the Nordic Network for Clinical Islet Transplantation (Olle Korsgren, Uppsala University, Sweden). Prior to the experiments, the human islets with the 70-

90 % purity had been cultured at 37◦C (5%CO2) for 1–5 days in CMRL 1066 supplemented with 10 mmol/l HEPES, 2 mmol/l L-glutamine, 50 μg/ml gentamicin, 0.25 μg/ml fungizone, 20 μg/ml ciprofloxacin ,10 mmol/l nicotinamide and 5.6 mmol/l glucose. The islets were then hand-picked under stereomicroscope prior to use.

Cell culture (papers III and IV)

INS-1 832/13 and INS1E cells were cultured in RPMI-1640 containing 11.1 mM D-glucose and supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100μg/ml streptomycin , 10mM HEPES, 2mM glutamine, 1 mM sodium pyruvate, and 50 μM β-mercaptoethanol , at 37º C in a humidified atmosphere containing 95% air and 5% CO2. After the initial culture period, the culture medium was replaced with medium containing 5.5 or 5 and 20 mM glucose, and culture continued for three days.

Human islets culture (papers I and IV)

Human islets (300 islets/30 mm Dishes) were in cultured RPMI media containing different glucose (5.5 or 5 and 20 mmol/l) in incubator 37°C, 5% CO2 for 72 hrs, in the presence or absence of diazoxide and GSK3β-inhibitor (CT) (paper IV). In one series of experiments islets from diabetic and non-diabetic subjects cultured at 5.5 mM glucose for 24 hrs in the presence and absence of Bt2-Camp (10 or 100 μmol/l) (paper I).

Rat islets culture (paper II)

250 islets from Wistar rat were in cultured RPMI media containing different glucose (5 or 16.7 mmol/l) in incubator 37°C, 5% CO_2 for 24 hrs, in the presence or absence of palmitate (100 or 1000 μmol/l) or rosiglitazone (1 μmol/l).

Insulin secretion from isolated islets and pancreatic β-cell lines (papers I-IV)

Freshly isolated islets or cultured islets were preincubated for 30 min at 37°C in Krebs Ringer bicarbonate buffer, pH 7.4, supplemented with N-2 hydroxyethylpiperazine-N'-2-ethanesulfonic acid (10 mmol/1), 0.1% bovine serum albumin, and 1 mmol/l glucose. Each incubation vial contained 12 islets in 1.0 ml KRB buffer solution and treated with 95% O_2-5% CO_2 to obtain constant pH and oxygenation. After preincubation, the buffer was changed. The islets were then incubated with different test agents for 1h at 37°C in a metabolic shaker (30 cycles per min). Concerning the experiments with INS-1 832/13 or INS1E cells, the cells were treated the same way as pancreatic islets but they were placed in H-chamber for the indicated time. Immediately after incubation an aliquot of the medium was removed and frozen for subsequent assays of hormone release.

Isolation of mitochondria (paper III)

Cultured INS1E cells at 5.5 or 20 mM glucose for 72 hrs were washed twice with Dulbecco's PBS and twice with ice-cold homogenization buffer (pH 7.4) consisting of 0.25 M sucrose, 0.25 Mm EGTA, 5 mM HEPES, 1 mM DTT and protease inhibitory cocktail. Cells were subsequently detached with a cell scraper and suspended in 5 mL of homogenization buffer. Cells (15 x 106) were disrupted by 15 strokes of a Teflon pestle in a 10-mL glass homogenizer and mitochondria were isolated as previously described.5 Homogenization and all subsequent steps were performed at 4oC. Mitochondria were banded just above the 10–30% interface and were collected by aspiration using a syringe. The mitochondrial fraction was diluted 1:1 in ice-cold PBS and centrifuged for 15 min at 14,000 rpm in a bench-top centrifuge to pellet the mitochondria. The mitochondria were subsequently washed twice in ice-cold PBS. Supernatants were discarded, and the mitochondrial pellets were then frozen in liquid nitrogen and stored at -80oC until analysis.

Biochemical and radioimmunological analysis

Determination of insulin, glucagon and somatostatin

The concentrations of insulin, glucagon, and somatostatin were determined by RIA [182]. The radioimmunoassay kits for insulin, glucagon and somatostatin were from Euro-Diagnostica, Malmö, Sweden.

Detection of mRNA expression by qPCR (papers I, II and IV)

RNA from islets was isolated using TRIzol (Invitrogen, Carlsbad, CA, USA) according to a modified RNA extraction protocol and from INS-1 832/13 cells by using RNAeasy (Qiagen, Hilden, Germany) and transcribed into cDNA as described elsewhere[183]. Concentration and purity of total RNA was measured with a NanoDrop ND-1000 spectrophotometer (A260/A280>1.9 and A260/A230>1.4) (NanoDrop Technologies, Wilmington, DE, USA) and RNA Quality Indicator (RQI) higher than 8.0 (Experion Automated Electrophoresis, Bio-Rad, USA) which could be considered as a high-quality total RNA preparation and thus giving a consistent extraction procedure. For performance of qPCR on a HT7900 system (Applied Biosystems, Foster City, CA, USA) we used the QuantiTect primer assays iNOS, PDE3A, PDE3B, GPR40, VDAC1, VDAC2, ChREBP and TXNIP and QuantiFast SYBR Green (Qiagen, Venlo, the Netherlands) and TaqMan (Applied Biosytems, CA, USA) PCR according to the manufacturer's instructions. The specificity of all primer assays was validated by melting curve analysis and gel electrophoresis. Gene expression relative to GAPDH or HPRT1 was calculated using the ΔΔCt method.

Western blot analysis (papers I- IV)

Human or rat islets (400 islets) and INS1 832/13 or INS1E cells (2x 10^5) were suspended in 100 µl of SDS-buffer (50 mM Tris-HCl, 1mM EDTA) supplemented with Complete protease inhibitor cocktail (Roche), frozen and sonicated on ice on the day of analysis. The protein content of the homogenates was determined according to the Bradford method.

Homogenate samples of islets and INS1 832/13 representing 30 µg of total protein were run on 7.5% SDS-polyacrylamide gels (Bio-Rad, Hercules, CA, USA). After electrophoresis, proteins were transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). The membranes were blocked in LS-buffer (10 mM Tris, pH 7.4, 100 mM NaCl, 0.1% Tween-20) containing 5% non-fat dry milk powder for 40 min at 37°C. Subsequently the membranes were incubated over night with the following primary antibodies: polyclonal rabbit anti-iNOS antibody (1:150), polyclonal rabbit anti-GPR40 antibody (1:150), rabbit-raised polyclonal anti-VDAC1 and goat-raised polyclonal anti-VDAC2 antibodies (1:500) at room temperature. After washing (three times) in LS-buffer the membranes were finally incubated with a horseradish peroxidase-conjugated antigoat and anti-rabbit antibodies (1:500). Immunoreactivity was detected using an enhanced chemiluminescence reaction (Pierce, Rockford, IL, USA). The results were quantified by densitometric analysis using the Bio-Rad software.

Immunofluorescence and confocal microscopy (papers I and II)

The islets were fixed with 4% formaldehyde, permeabilized with 5% Triton X-100, and unspecific sites were blocked with 5% Normal Donkey Serum (Jackson Immunoresearch Laboratories Inc, West Grove, PA, USA). The expression of iNOS was detected with a polyclonal antibody (1:50) (Abcam, UK.) in combination with Cy3-conjugated anti-rabbit IgG (1:100) (Jackson Immunoresearch Laboratories Inc). GPR40 was detected with a polyclonal antibody (1:50) in combination with Cy3-conjugated anti-rabbit IgG (1:100) (Jackson Immunoresearch Laboratories Inc. West Grove, PA, USA). The GPR40 antibody was raised in rabbits against the C-terminal peptide: NH2-CVTRTQRGTIQK-COOH (Innovagen, Lund, Sweden). For staining of insulin, glucagon and somatostatin islets were incubated with a guinea pig-raised anti-insulin antibody (1:1000) (Eurodiagnostica, Malmö, Sweden), guinea pig anti-glucagon (1:500) and rat anti-somatostatin antibody (1:250) (both from Jackson Immunoresearch Laboratories), followed by an incubation with a Cy5-conjugated anti-guinea pig IgG antibody and anti-rat IgG antibody (Jackson Immunoresearch Laboratories Inc) (1:150). The fluorescence was visualized with a Zeiss LSM510 confocal microscope by sequentially scanning at (excitation/emission) 543/570(Cy3) and 633/>650 nm (Cy5).

cAMP measurement (paper I)

For the measurement of cAMP human islets from non-diabetic or diabetic donors were incubated for 60 min at 1 or 16.7 mmol/l glucose. Each incubation vial contained 50 islets in 1.0 ml of Krebs-Ringer bicarbonate (KRB)-buffer. After the incubation the islets were treated with 100 mM HCl for 5 min and then frozen. At the day of analysis samples were sonicated on ice and cAMP was measured using a cAMP EIA kit (Cayman Chemical Company) according to the manufacturer's instructions.

Nitrite measurement (paper I)

After culture of diabetic and non-diabetic islets at 5.5 mmol/l in the presence or absence of Bt_2-cAMP (10 or 100 µmol/l) for 24 hrs or culture of normal islets at 5.5 mmol/l or a high glucose concentration (20 mmol/l) for 72 hrs. The released nitrite in the culture medium was determined according to the Griess method by using a commercial available Colorimetric Assay Kit following the manufacturer's instruction (Cayman Chemical Company).

Cell viability (papers I, III and IV)

Cell viability was measured either on dispersed human islet cells when the islets were subjected to different experimental conditions e.g. down-regulation of VDAC1 or VDAC2 or when the islets were cultured at high glucose in the absence or presence of different test agents (papers I and IV). Cell viability assay was also performed on the INS-1 832/13 or INS1E after exposing the cells to high glucose (20 mmol/l) in the absence or presence of GSK3β-inhibitor (CT) (paper IV). Measurement of cell viability was performed using the MTS reagent kit according to the manufacturer's instructions (Promega).

Cell proliferation assay (paper IV)

INS-1 832/13 β-cells were seeded at 1x 10^4 cells/well into 48 well plates in RPMI-1640 medium. The cells were transfected with the Vdac1 and Vdac2 siRNA at a final concentration of 50 nmol/l or with control siRNA (non-

targeting siRNA) for 24h at the same concentration before changing to fresh media including antibiotics. After transfection plates were incubated for 2-6 days, in 37°C, 5% CO_2. Both control and transfected cells were counted on consecutive days in a Bücker chamber under stereomicroscope.

Oxygen consumption (paper IV)

The oxygen consumption rate (OCR) was measured in INS-1 832/13 cells using the XF (extracellular flux) analyser XF24 (Seahorse Bioscience), as previously described in detail [184]. An assay medium composed of 114 mM NaCl, 4.7 mM KCl, 1.2 mM KH2PO4, 1.16 mMMgSO4, 20 mM HEPES, 2.5 mM CaCl2, 0.2% bovine serum albumin, pH 7.2, and supplemented with 2.8 mM glucose was used in the XF analysis. The cells were seeded in an XF24 24-well cell culture microplate at 250,000 cells/well (0.32-cm2 growth area) in 500 µl of growth medium and incubated overnight at 37 °C in a humidified atmosphere of 95% air and 5% CO2. Prior to assay, RPMI 1640 medium was removed and replaced by 750 µl of assay medium. The cells were preincubated under these conditions for 2 h at 37 °C in air. The experiments were designed to determine respiration in low (2.8 mM) glucose and for 60 min following the transition to high (16.7 mM) glucose. The proportions of respiration driving ATP synthesis and proton leak were determined by the addition of oligomycin (4 µg/ml). After a further 30 min, 4 µM of the uncoupler carbonyl cyanide-p-trifluoromethoxyphenylhydrazone was added to determine maximal respiratory capacity. After a further 10 min, 1 µM rotenone was added to block transfer of electrons from complex I to ubiquinone.

Two dimensional gel electrophoresis (2DE) (paper III)

The mitochondrial pellets were re-suspended in 100 µL of 1% Triton X-100 and 2% SDS and solubilized by water-bath sonication. The samples were incubated at 4^{o}C for 30 min and treated with PlusOne 2-D Clean-Up Kit (Amersham Biosciences). The protein pellet was resuspended in lysis buffer for the isoelectric focusing and protein concentration was determined using 2-D Quant Kit (Amersham Biosciences). Solubilization of protein samples and rehydration of IPG strips were performed essentially. The rehydrated strips were focused on the Protean IEF Cell (Bio-Rad) for about 35 kV h at a maximum of 8kV in rapid voltage ramping mode with a

maximum current per strip of 30 μA. Equilibrationand transfer of the IPG strips to the second dimension was done. The SDS-PAGE was performed on 8–16% precast polyacrylamide gels (Criterion Gel System, BioRad). The gels were run at room temperature with a constant voltage of 120V for 10 min, followed by 200V for 60 min.

Mass spectrometry (MS) (paper III)

Forty-four unknown spots with consistent presence in 2-D analytical gels of replicate groups were selected and excised manually from the gels. One protein-free gel piece was treated in parallel as a negative control. Individual gel spots were subjected to in-gel trypsin digestion. Protein digests were analyzed by ESI-LC-MS/MS using a high-capacity iontrap (HCTplus™, Bruker Daltonics, Germany) tandem mass spectrometer. Liquid chromatography was performed using an Ultimate™ (LC-Packings, Dionex, Netherlands) system equipped with a Famos™ auto sampler. Samples were concentrated on a trapping column (300 μm i.d., 1 cm, LC-Packings) at a flow rate of 25 μL/min. For the separation with a C18 PepMap column (75 μm i.d., 10 cm, LC-Packings) a flow rate of 200 nL/min was used. For electrospray ionization, we used coated silica Picotips™ (New Objective, MA, USA). Instruments were controlled using HyStar™ 3.0 and EsquireControl™ 5.2 software (Bruker Daltonics). The sample injection volume used in all experiments was 5 μL and tuning parameters of the mass spectrometer were kept as constant as possible. Individual MS/MS spectra were searched against Swissprot/NCBInr using Mascot™ software (Matrixscience, London, UK). The interpretation and presentation of MS/MS data was performed according to published guidelines. The peptide and fragment mass tolerances were 2.5 and 0.8 Da, respectively. Identification was based on the presence of at least two peptides and on Mascot scores of >50.

Transfection of cells and human islets (paper IV)

Full-length cDNA encoding prevalidated Vdac1 construct was purchased from Source BioScience imagenes (OCAAo5051H1165-pDEST26), Berlin. INS1 832/13 cells were seeded in six-well plates at a density of ~5 x 105 cells in culture media without antibiotics and transfected with Effectene Transfection Reagent (Qiagen; cat. no 301425) according to the manufacturer's instructions

For Vdac1 and Vdac2 small interfering RNA (siRNA) experiments, 20-25-nucleotide stealth-prevalidated siRNA duplex designed for rat Vdac1 and Vdac2 (Applied Biosystems) were used while isolated human islets were transiently transfected using lentiviral based shRNA-silencing technique (Santa cruz, CA, USA). The cells and islets were transfected with DharmaFECT® 1 (Dharmacon; Lafayette, CO, USA) according to the manufacturer's instructions. Experiments were performed 48h after transfection unless specified differently.

Statistical analysis (papers I- IV)

Probability levels of random differences were determined by analysis of variance followed by Tukey-Kramer's multiple comparisons test. Student's unpaired t-test was also used to detect the level of the significance for the difference between sets of data. Results were expressed as means ± SEM. $P < 0.05$ was considered statistically significant.

Results and Discussion

Paper I

Prolonged exposure of pancreatic β-cells to elevated glucose concentrations in vitro or chronic elevation of blood glucose (hyperglycemia), is well known to contribute to the development of impaired GSIS and β-cell apoptosis [185-187]. This process is often called glucotoxicity, is apparently involved in the pathogenesis of type 2 diabetes [167, 185]. Although the exact mechanisms behind glucotoxicity remain unknown, the induction of chronic oxidative stress has been implicated as an important contributing factor [167, 188]. Indeed free radicals like NO may well contribute to the observed process behind the oxidative stress. Furthermore, NO derived from iNOS has been proposed as a mediator of immune-induced β-cell damage in type 1 diabetes [161]. However, the expression of islet iNOS and its effect on pancreatic β-cell function and survival in type 2 diabetic subjects were not determined and thus have been investigated in this work.

Using different approaches (confocal microscopy, western blot and qPCR), we found a rich occurrence of iNOS protein in islets isolated from human type 2 diabetic subjects. This observation might consider chronic hyperglycemia as a main contributing factor in the development of non-immunogenic diabetes by inducing iNOS in the pancreatic islets. This is in line with previous findings, indicating that impaired glucose stimulated insulin secretion is associated with abnormal production of NO in the GK rat (animal model of type 2 diabetes) islets [189, 190], and comparable to what has been detected in transgenic mice overexpressing iNOS in pancreatic β-cell [191]. In the present investigation, we also detected a markedly increase in the expression of iNOS and nitrite production in non-diabetic subjects after culturing the islets at 20 mM glucose (high glucose) for 72 h. this is in agreement with previous findings showing that incubation of rodent islets at high glucose results in iNOS expression [159, 192].

Earlier studies have demonstrated that a protective mechanism against NO-mediated impairment of β-cell function might be exerted through the cAMP system [163, 192]. A study conducted by our group [190], revealed that GLP1, potent cAMP-generating incretin, counteracted the impaired insulin response to glucose in the GK rat islets in association with a marked suppression of iNOS expression and activity. Inhibitory action of GLP1 on iNOS expression was abolished by PKA inhibitor H-89, showing the importance of cAMP system in this context [190]. Along this line of thought, we found the islets from type 2 diabetic subjects have low cAMP content after incubation in high glucose (16.7 mM) compared with the non-diabetic controls. Interestingly, it is established that the suppression of cAMP degradation through inhibiting islet PDE activities stimulates GSIS [101]. This prompts us to investigate the role of different PDEs and whether the suppression of iNOS by activating cAMP system is associated with increase insulin secretion in type 2 diabetic islets. Among the different PDEs found in islet tissue PDE3 is most important in relation to regulation of insulin secretion [101]. We selected the membrane permeable cAMP analogue Bt_2cAMP and different inhibitors of PDEs like, rolipram, zaprinast, milrinone, IBMX and cilostazol. Our data showed that cAMP analogue induced a marked suppression of iNOS, a pronounced decrease of medium nitrite levels as well as an increased insulin release in islets cultured for 24h from type 2 diabetic subjects. Moreover, we found also that short-term incubation (1h) of islets after culture at 5.5 mM glucose with Bt_2cAMP revealed an increased insulin response to high glucose being comparatively more pronounced in diabetic islets than non-diabetic relative to solvent control cultured without cAMP analogue. Among the PDEs inhibitors, we found cilostazol to be more potent than others in the potentiation of GSIS and its effect was greater in diabetic vs non diabetic islets. This finding is supported by the fact that we could find an eightfold increase in mRNA expression of PDE3A and a threefold increase in PDE3B in diabetic islets compared with non-diabetic. So PDE3 inhibition is a promising target for relieving the impaired insulin secretion in type 2 diabetes.

Notably, we found iNOS protein to be expressed in glucagon-producing α-cells from the islets of type 2 diabetic subjects, similar to what has been detected in the GK rat [190]. Previous studies have found NO to be a potent glucagon Secretagogues [163, 190], so the abnormal expression of iNOS in the α-cells might contribute to abnormal glucagon hypersecretion in human type 2 diabetes. This idea is further supported by our findings that revealed abnormal increased glucagon secretion in type 2 diabetic islets after

incubation at high glucose. However, it must be kept in mind that islet NO production must be considered to reflect mainly the greater number of β-cells, and a more evidence for the role of α-cells in NO-glucagon interaction remains to be further clarified.

The various mechanisms involved in the initiation and progression of β-cell dysfunction in type 2 diabetes remain disputed [193]. However, previous studies have demonstrated, by using inhibitors of different NOS isoenzymes or NO scavengers, the pivotal importance of increased production of nitrogen reactive species, for instance, NO and peroxynitrite in the initiation of β-cell injury[192, 194, 195]. Moreover, it has been shown, that the intracellular NO donor hydroxylamin concentration-dependently inhibits GSIS while IBMX-stimulated insulin release in fact is modestly enhanced by low concentrations of the NO donor [196]. Induction of iNOS has been suggested to be an important determinant of the progression to a glucotoxic and/or inflammatory state with concomitant β-cell dysfunction [159, 190]. Interestingly, inhibition of iNOS induction has been implicated in the protection of β-cells exposed to cytokines and attenuation of the deleterious effect of NO on important cellular signaling pathways [197]. This in line with our experiments, using specific iNOS inhibitor aminoguanidine which reversed the deleterious effect of glucotoxic condition after culturing the isolated normal human islets for 72h at high glucose (20mM) and restored completely the impaired insulin release back to normal. This is in accordance with what was recently shown that iNOS gene silencing in cytokine-treated human islets in vitro results in increased GSIS [198]. Notably, the defective insulin response in type 2 diabetes is multifactorial in origin and isn't fully corrected by cAMP or PDE3 inhibitors. However our data suggest that cAMP-activated signaling pathway inducing insulin release is fully operating in islets from human type 2 diabetes. This is in analogy with what has been found in GK rat [189, 190].

In conclusion, the present data show that abnormally increased expression of islet iNOS coincide with pancreatic β-cell dysfunction suggesting that excessive NO production might be an important contributing factor for the dysfunction of both α- and β-cells seen in human type 2 diabetes. PDE inhibitors that target the degradation of selective cAMP compartments of importance for the regulation of iNOS might counteract these abnormalities and restore β-function, at least in part by activating cAMP/PKA system. Incidentally, the PDE3 inhibitor cilostazol has recently been used in clinical trials for other purposes, that is, prevention of cerebral ischemia [199].

Hence, the present novel data hopefully pave the way for new therapeutic interventions to reduce islet NO production among which islet cAMP compartments seems to be a useful approach.

Paper II

With the discovery of GPR40 as a receptor for FFAs and the established effects of FFAs in the augmentation of GSIS, we wanted to study the importance of GPR40 for this process more closely as well as the impact of long-term hyperglycemia on the expression level of GPR40 in the islets. For this purpose we used two different animal models. The Zucker Diabetic Fatty Rat (ZDF) represents a good model of obesity and diabetes and the Goto-Kakizaki (GK) rat represents a state of hyperglycemia and glucotoxicity without weight gain.

The present data demonstrate strong evidence for a causal relationship between the expression level of GPR40 and palmitate-induced modulation of insulin, glucagon, and somatostatin secretion. Using confocal microscopy, we detected the expression of GPR40 not only in the insulin and glucagon cells but also in the somatostatin cells. Moreover, we found that the islets of GK rat express a very low abundance of GPR40 in all three kinds of their pancreatic endocrine cells compared with the control. Interestingly, the reduction in GPR40 expression in the islets of GK rats was coexisted with abolished response of these islets to palmitate stimulus. This is in line with what has been detected in GPR40-knockout mice [90, 200]. The decreased expression of GPR40 in the GK rats could not be attributed to any major changes in the contribution of β-cells to islets mass, since the young GK rats (6-8 weeks) show a normal content of insulin in their islets [201], as well as the serum TG, cholesterol, HDL and LDL levels were almost of the same level as in Wistar controls [202] and their hyperglycemia is associated with a deficient insulin response to glucose [190]. Although the islets of GK rats normally display a wide variety of defects [189, 201, 203], an adequate expression of GPR40 seems to be of extreme importance for the proper functioning of islet cells in response to FFA. So the reason for the extremely low expression of GPR40 in the GK islets is presently unclear, but it is conceivable that it might be a consequence of the high blood glucose levels.

In contrast to GK islets, the islets of the young fa/fa rats, a model of obesity secondary to genetic unresponsiveness to leptin [204] express a markedly increased expression of GPR40 and we suggest that this strongly contributes to the effects of the elevated FFA levels in the induction of hypersecretion of insulin and glucagon and the suppression of somatostatin release found in this animal. Our fa/fa rats were normoglycaemic whereas blood FFA, TG, cholesterol, and HDL were slightly elevated and LDL was slightly reduced [205]. An exaggerated insulin response to enhanced circulating levels of FFA by islets of ZDF rat seems to be a possible explanation to why these rats become rapidly obese. Our present data is in analogy with a recent study showing a weak cause-effect relationship between the enzymes involved in islets FFA oxidation and FFA-stimulated insulin release [88, 206].

Increased plasma concentrations of glucose are associated with defects exerted by the increased metabolic and secretory activities of the beta cells [159, 201]. The subsequent oxidative stress induced by the metabolic and secretory pathways, has been implicated in the induction of an abnormal β-cell function [207]. However the underlying mechanisms for the lower expression of GPR40 in GK islets and its higher expression in ZDF islets are unclear and remain to be explored. In order to differentiate between genetically causative factors in contrast to environmental factor *i.e.* a high concentration of glucose or FFA, we found that culture of normal Wistar islets for 24 h at high glucose (16.7 mM) almost completely suppressed the expression of GPR40 compared with islets cultured at low glucose (5.5 mM). This confirms that not only long-chain FFA but also glucose seems to have a marked regulatory influence on GPR40 expression. Moreover, we have demonstrated that the acute stimulatory effects of palmitate on insulin secretion is markedly reduced in normal Wistar islets cultured at high glucose thus being in accordance with marked suppression of GPR40 in their β-cells. Our data also showed that islets cultured with low concentration of palmitate displayed increased mRNA level of GPR40 while at high concentration, the expression tended to decrease below the control level. Furthermore, we found that GPR40 protein expression was increased to the same level both at low and high palmitate concentrations. We suggest that posttranslational modification at the GPR40 level might have a vital role in regulating palmitate-induced hormone secretion.

Finally, another most prominent finding of the present study is that the total suppression of GPR40 protein would influence not only insulin release induced by palmitate but also that induced by glucose. We found that when different groups of cultured normal Wistar islets were challenged with high glucose. The stimulatory effects of glucose on insulin secretion is lower in high glucose-cultured control groups compared with groups cultured with ROZ, palmitate or both which displayed GPR40 protein expression. These data support a particularly important role of high glucose to suppress the GPR40 expression and mildly increased FFA to stimulate an increased expression of the receptor.

In conclusion, by using different approaches, we showed that FFA-stimulated insulin and glucagon as well as FFA-induced suppression of somatostatin is highly dependent on and correlated to the extent of GPR40 expression being regulated by both FFA and glucose, from two highly animal models of T2D.

Paper III

Proteomics denotes the study of the proteome, which includes listing all the protein present in a cell or tissue type at a specific time under specific condition, quantitation and functional characteristics of these proteins to interpret their relationships [208]. Such global protein expression profiling is valuable compared with single gene or protein regulation studies since changes in several functional groups are observed at the same time. The objective of current study was to investigate changes in the mitochondrial protein expression of INS-1E cells that have reduced GSIS after long-term exposure to high glucose levels. Earlier study on INS1E mitochondrial proteomics showed 34 differentially expressed protein spots in response to high glucose but without any protein identification [209]. The present proteomic analysis showed detection of 33 different proteins corresponding to 58 differentially protein spots. It is beyond the scope of this study to discuss in detail the possible role of all identified proteins in islet pathophysiology. However, studying the expression patterns of some proteins might provide the possibility to address the mechanisms underlying β-cell dysfunction resulting from glucotoxicity.

In the current study, two-dimensional gel electrophoresis (2DE) showed increased expression of GPR75, HSP60, and HSP10 in mitochondrial fraction of INS1E cells cultured at high glucose (20mM) for 72h, which may indicate the adaptation of the β-cells for survival in glucotoxic condition. Overexpression of GPR75 has been implicated in the prolongation of lifespan of human fibroblast [210]. HSP10 and HPS60 were found to play regulatory role in protein folding/unfolding, protein degradation, anti-oxidative stress and anti-apoptosis [211, 212].

Chronic exposure of pancreatic β-cells to high glucose induces the increased expression level of proapoptotic proteins like, Bax, p53, Fas, as well as increased mitochondrial cytochrome release into cytosol [213]. In this regard it has been documented that Bax and Bak accelerate the opening of VDAC and allow cytochrome c to pass through the channel [214]. VDAC1 overexpression has been involved in the induction of apoptotic cell death in mouse, rat and human [180]. In a previous study [213], despite the fact that in glucotoxicity Bax induced apoptogenic mitochondrial cytochrome c release through its binding with VDAC, no significant change in the expression of VDAC was detected in pancreatic β-cells exposed to high glucose. Contrarily, previous study has shown two up-regulated VDAC1 spots on 2-DE analysis of islets from streptozotocin-treated diabetic mouse [215]. These studies support the role of VDAC1 as a crucial factor in the process of mitochondria-mediated β cell apoptosis. Moreover, in our study, 5 spots with similar molecular mass but different p*I*s values were identified as VDAC1. We found two spots of VDAC1 disappeared in response to high glucose (20mM), expression of one VDAC1 spot was increased three-fold and the two other VDAC1 spots were overexpressed on the 2-D gel of high glucose exposed INS1E cells without any significant association. Changes in expression of a single isoform (spots) of a protein on 2-DE do not necessarily signify alteration in total protein amount. This idea is supported by western blot experiments that revealed overexpression of VDAC1in INS1E cells exposed to high glucose. This finding of increased expression of VDAC1 suggests increased susceptibility to apoptotic cell death in response to high glucose challenge. This suggestion is further supported by downregulation of VDAC2 as evidenced on 2-D gel as well as by western blotting since VDAC2 is known to interact with and inhibit proapoptotic protein Bak and VDAC-2-deficient cells were shown to be more susceptible to apoptotic death [216].

It has been documented that chronic exposure of pancreatic β-cells to high glucose decreases the glucokinase binding to VDAC which eventually results in decreased ATP-dependent insulin release [213]. In this context, we also found the expression of spots corresponding to ATP synthase α, β, and δ chains were significantly decreased on the 2-D protein map of mitochondrial fraction of high glucose treated INS1E cells, suggesting that the failure of glucose to stimulate insulin release is conceivably due to reduced ATP generation. It is conceivable that the alteration in the VDAC1 and VDAC2 expression could represent a consequence of early step in the β-cell dysfunction that could be an important target process to prevent impaired insulin secretion and β-cell apoptosis.

In summary, these changes in protein expression pattern can be correlated to the altered phenotype of β-cells and may interpret cellular dysfunction in glucotoxicity resulting in altered insulin secretion. However Functional studies will be required to pinpoint the mechanisms by which they impair islet function. The present data also revealed for the first time that chronic exposure of INS1E cell to high glucose resulted in increased expression of VDAC1 and decreased VDAC2 expression. VDAC1 and VDAC2 might potentially participate in the evolution and persistence of deleterious effects of glucose on β-cell function during hyperglycemic episode, although the precise interactive mechanisms remained to be clarified.

Paper IV

It is well established that VDAC regulates the energy balance of mitochondria and the entire cell by providing the pathway for transporting various metabolites and nucleotides like ATP/ADP into and out of the mitochondria. So it has a very important role in the regulation of cell function and viability. Combined with the results from our previous study (paper III) that chronic exposure of INS1E cells is accompanied with a marked expression of the VDAC1 and a reduction of VDAC2 by proteomic analysis, we proposed a hypothesis that up-regulated VDAC1 and down-regulated VDAC2 expression may be involved in pancreatic β-cell dysfunction and apoptosis. We therefore wanted to explore the functional link between simultaneous alteration in VDAC1/VDAC2 expression and pancreatic β-cell dysfunction in the pathogenesis of T2D.

Since mitochondrial dysfunction has been implicated in the development of defective insulin release in T2D [217]. In present study, we pinpointed the changes in the expression level of two outer mitochondrial proteins called VDAC1 and VDAC2. It has been demonstrated that VDAC is a critical player in apoptosis and the contribution to the function of mitochondria in cell life and death [173, 214]. Our results using human pancreatic islets demonstrated for the first time that chronic exposure (72h) of pancreatic islets to high glucose concentration (20mM) caused up-regulation of VDAC1 expression and downregulation of VDAC2 expression showing that an altered expression of VDAC1 and VDAC2 might be associated with defective insulin secretory response of pancreatic β-cells observed during hyperglycemic episode. This is similar with what we found in the human type 2 diabetic islets which also showed a similar alteration in the VDAC1 and VADC2 expression.

Sargsyan [218] has shown that diazoxide (K^+-channel opener) can improve β-cell function via intermittent inhibition of insulin secretion a process called β-cell rest, and by the reduction of ER stress induced by continuously activated exocytotic machinery during chronic exposure of pancreatic β-cells to high glucose. Along this line of thought, we found that diazoxide has only marginal effect on the expression of VDAC1 and VDAC2 in the human islets cultured for 72h at high glucose (20 mM), suggesting that the observed changes in the expression of these two anion channels could be associated with confounding effects of a sustained hyperglycemia and not related to the increased secretory property of β-cells induced by high glucose. On the other hand, we assessed the VDAC1 and VDAC2 mRNA and protein levels in the presence of GSK3β-inhibitor (CT) and it was found that GSK3β-inhibitor greatly restored the altered expression of VDAC1 and VDAC2 induced by long-term culture of β-cells at high glucose concentration. This was concomitant with a greatly improved β-cell viability and insulin secretion. Elimination of GSK3β has been associated with increased rat neuronal cell survival [219]. Moreover, elevation of GSK3β activity has been shown to be associated with a marked reduction of β-cell proliferation and increased apoptosis [220, 221]. Our results showed that the signals produced downstream of GSK3β upon activation are of importance for the observed effect on the mitochondrial VDAC1up-regualtion and VDAC2 down regulation during hyperglycemia.

Furthermore, we found that overexpression of VDAC1could be harmful to the β-cells since the transfected INS-1 832/13 cells with VDAC1 construct showed both reduced basal and also defective glucose-stimulated insulin release. This was concomitant with a decrease in proliferation in VDAC1 overexpressing INS-1 832/13 cells compared to the control cells. This is in agreement with a previous study that has shown up-regulated VDAC1 expression from streptozotocin-treated diabetic mouse islets [215]. While RNAi mediated knockdown of VDAC1 expression did not affect the basal and glucose-stimulated insulin secretion as well as cell proliferation. This could be explained by the fact that the expression of VDAC1 is very low in the normal physiological condition and only increased during hyperglycemia. Moreover, to assess the functional consequences of VDAC2 down regulation. We used also RNAi based approach in INS-1 832/13 cells. A 70% reduction of VDAC2 mRNA levels, paralleled by a strong decrease in VDAC2 protein levels, caused a 50% suppression of both basal and glucose stimulated insulin release. This was accompanied with a decrease in cell proliferation when the INS-1 832/13 cells were cultured for an additional period after down regulation. These data suggest a different functional property of VDAC1 and VDAC2 in the regulation of β-cell function and highlight the deleterious effect of VDAC1 overexpression in this regard.

Previous studies have demonstrated that high glucose induces the expression of both ChREBP and TXNIP transcriptional factors in pancreatic β-cells [222]. This is in accordance with what we found in human type 2 diabetic islets which showed increased expression of ChREBP and TXNIP. Overexpression of ChREBP and TXNIP has been shown to lead to induction of apoptosis by either directly up regulating the expression of certain apoptotic proteins or indirectly by increasing the activity of downstream target genes [222, 223]. Therefore we particularly were interested whether ChREBP and TXNIP expression level could have any impact on VDAC1 expression. We found that knockdown of the ChREBP expression in INS-1 832/13 cells by RNA interference was associated with a decreased capacity of high glucose to increase VDAC1 expression during long-term culture of the cells at high glucose. Furthermore, RNAi mediated down regulation of TXNIP expression with preservation of ChREBP in the cells prevented high glucose-induced expression of VDAC1, suggesting that TXNIP is essential for the ChREBP mediated signaling pathway to overexpress VDAC1 in the cells. However,

TXNIP is capable of influencing numerous signal transduction pathways, as previous study has shown that TXNIP inhibits thioredoxin activity and thereby reduces the cellular antioxidant capacity and promotes ROS production [224]. It has been documented that ROS can trigger apoptosis via VDAC-dependent permeabilization of the OMM [225]. On the other hand, TXNIP knockdown has been shown to improve glucose tolerance and insulin sensitivity in animal model of type 2 diabetes via inhibition of glucose-induced pancreatic β-cell apoptosis [226]. This is in line with our present data that suppression of VDAC1 expression, which could be induced by downregulation of TXNIP, increased β-cell viability and function. Interestingly, 2-Deoxy-D-glucose which is a substrate of the first enzyme in the glycolytic pathway was found to induce increased expression of VDAC1after the culture period of INS-1 832/13 cells at basal glucose concentration indicating that the phosphorylation by hexokinase is sufficient to increase the VDAC1 transcription. It has been reported that resveratrol inhibits both mitochondrial ROS production and permeability transition thereby protecting the key intracellular organelle against the oxidative stress [227]. In this study, resveratrol was found to suppress high glucose-induced expression of VDAC1 after long-term culture of INS-1 832/13 cells at 20 mM glucose. Moreover, using human islets we found also that resveratrol protects effectively against glucotoxicity-induced beta-cell apoptosis when it was present during culture period. These results are comparable to those reported by previous studies [228, 229].

Finally, to explore whether these observed changes in the expression of VDAC1 and VDAC2 impacted mitochondrial activity, we analyzed oxygen consumption rate (OCR) in the presence of overexpression of VDAC1 and downregulation of VDAC2 and we found overexpression of VDAC1 and downregulation of VDAC2 to be ineffective in stimulating OCR suggesting that the altered expression of VDAC1 and VDAC2 is associated with mitochondrial dysfunction in the β-cell.

In conclusion, our results indicated that alteration in VDAC1 and VADC2 expression may contribute to β-cell dysfunction and cell death in type 2-diabetes. However, additional studies are warranted to establish the role of up- and down-stream signaling pathways upon overexpression of VDAC1 or downregulation of VDAC2 in pancreatic β-cells and also to establish therapeutic potentials of targeting VADC1 and VDAC2 to prevent β-cell dysfunction and apoptosis.

Concluding Remarks

In this thesis, the effects of glucotoxicity on pancreatic β-cell function have been investigated as illustrated in **Fig 4**. The following major conclusions were reached:

- ✓ cAMP/PKA system plays a central role in the suppression of abnormally increased iNOS expression through their stimulatory effects in certain cAMP-compartments, resulting in improved β-cell function in islets from T2D.
- ✓ GPR40 is abundantly expressed in pancreatic islets and modulates palmitate-induced secretion of insulin, glucagon, and somatostatin. A mild hyperlipidemia increases GPR40 expression and palmitate-induced effects on hormone secretion, whereas chronic hyperglycemia abrogates GPR40 expression and abolished palmitate-induced secretory effects.
- ✓ Coordinated changes in expression patterns of INS-1E mitochondrial proteins, in particular VDACs, may explain cellular dysfunction in glucotoxicity resulting in impaired insulin secretion.
- ✓ Altered expression of VDAC1 and VDAC2 possibly via an increased GSK3β activity and nitrosative cell stress signals seems to be a key component in the pathogenesis of glucotoxicity and it may explain β-cell dysfunction in glucotoxic and associated diabetic state resulting in impaired insulin release.

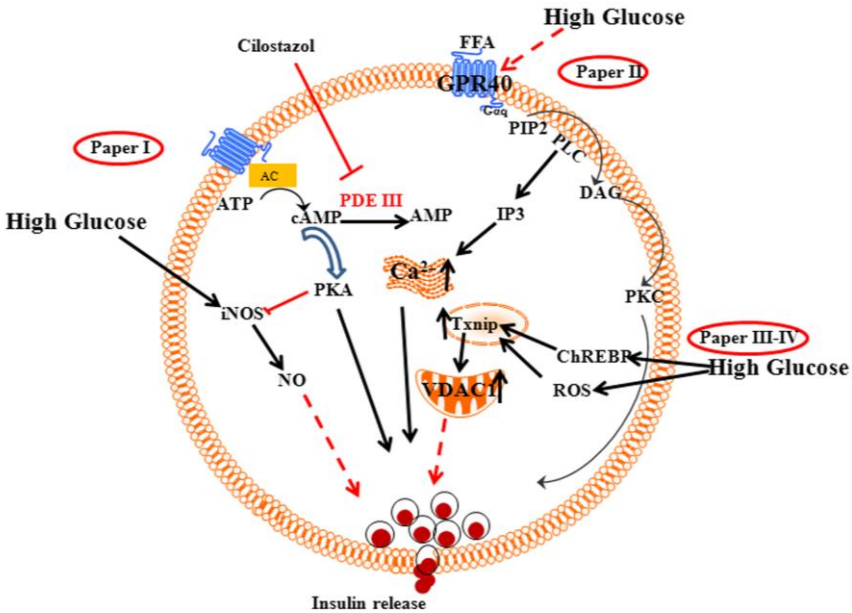

Figure.4 **Model of factors that influence insulin secretion under glucotoxic state.**

Populärvetenskaplig Sammanfattning

Typ 2-diabetes mellitus ökar över hela världen med en trend av minskande debutålder. Sjukdomen är ett komplext natur som kan ses som en rad olika metabola sjukdomar med delvis lika symtom, men med helt olika bakgrundsorsaker. Gemensamt för alla varianter av diabetes är att blodsockret är förhöjt även vid fasta. Typ 2-diabetes kännetecknas av insulinresistens och en progressiv förlust av insulinfrisättning från de pankreatiska β-cellerna. Förmågan att utsöndra tillräckliga mängder insulin bestäms av den funktionella kapaciteten hos pankreatiska β-celler och deras totala massa.

Glukos, eller druvsocker, är den huvudsakliga regulatorn av insulinproduktion och dess utsöndring i blodet.Om glukoskoncentrationerna är förhöjda över en längre period, har detta negativa effekter på β-cellfunktion, som sammanfattas med begreppet "glucotoxicitet". Denna leder till metaboliska avvikelser i β-cellen som endast är delvis kartlagda, men klart är att glukotoxicitet förknippas med ökad basal insulinfrisättning, minskad respons på stimuli att utsöndra insulin, och en gradvis utarmning av insulinproduktion. Glukotoxicitet leder också till minskad β-cellmassa genom induktion av apoptos, om denna inte kompenseras av en kompensatorisk ökning av β-cellproliferation och neogenes (nybildning).

Hos alla typ 2-diabetiker ser man, trots aggressiv läkemedelsbehandling, med tiden en obeveklig nedgång i den insulinproducerande β-cellens funktion. Nedgången beror åtminstone delvis på induktion av vissa enzymer som ökar produktionen av en rad reaktiva molekyler som kväveoxid (NO) och reaktiva syreradikaler (ROS), vilka är etablerade stressfaktorer och påverkar β-cellerna negativt.

Mitokondrierna är centrala i energiomsättningen genom att tillverka energirika föreningar såsom ATP och GTP, och helt oundgängliga för optimal β-cellsfunktion. Hur glucotoxicitet påverkar mitokondriefunktionen är inte känt i detalj.

Med hjälp av olika tekniker såsom immunohistokemi, Western blot, kvantitativ PCR, masspektrometri, cellviabilitet- och hormon-analys, har jag undersökt de mekanismer som kan vara av intresse och presenterat våra senaste fynd angående de strukturella och funktionella förändringar som

glucotoxicitet leder till i β-celler. Obehandlade kan dessa förändringar leda till β-cellsdysfunktion som kommer att kulminera i diabetes mellitus typ 2.

Beskrivning av delarbete 1-4

I den första studien upptäckte jag ett onormalt ökat uttryck av det inducerbara enzymet kväveoxidsyntas (iNOS) i pankreatiska öar från typ 2 diabetiska donatorer samt från öar som utsattes för kronisk hyperglykemi (in vitro). Induktionen av iNOS leder till produktion av mycket höga nivåer av NO som är toxiskt för β-cellen. Vi föreslår att detta kan vara en bidragande faktor för β-cell dysfunktion som ses vid typ 2-diabetes (T2D). Jag visade också att en ökning av den intracellulära signalmolekylen cAMP kunde motverka induktionen av iNOS och åtminstone delvis återställa β-cellsfunktionen genom aktivering av cykliskt AMP/PKA-systemet.

I den andra studien fann vi att GPR40, en membranbunden receptor som binder vissa fettsyror, är rikligt uttryckt i de Langerhanska ö-cellerna hos råtta och att GPR40 är av stor vikt vid reglering av insulin-, glukagon- och somatostatinutsöndring medierad av fettsyran palmitat. En mild hyperlipidemi ökar GPR40 uttrycket i de pankreatiska ö-cellerna hos råtta. Hyperglykemi har motsatt effekt och nedreglerar uttryck av GPR40 och motverkar palmitat-inducerad hormonsekretion. Vidare fann vi också att GPR40 är lågt uttryckt i pankreatiska ö-celler från den diabetiska råttstammen GK. Vi tror att GPR40 protein uttrycket påverkas olika av både FFA och glukos och är en lovande måltavla för läkemedelsbehandling (GPR40-antagonister och agonister) inom olika varianter av typ 2-diabetes.

I de tredje och fjärde delstudierna fann vi att kronisk glukosexponering av insulinproducerande β-celler (INS-1E och INS-1 832/13 celler) eller isolerade humana pankreatiska öar är förknippad med ökad nivå av det mitokondriella kanalproteinet VDAC1, medan den liknande molekylen VDAC2 har minskat uttryck. Samtidigt observerades en tydlig minskning av glukosstimulerad insulin-frisättning (GSIS). I likhet med dessa experiment, visade pankreatiska öar tagna från typ 2-diabetiska donatorer ett ökat uttryck av VDAC1 och ett minskat uttryck av VDAC2. Jag fann också att uttrycket av VDAC1 kunde vara direkt eller indirekt under kontroll av transkriptionsfaktorerna ChREBP och TXNIP. Denna idé stöddes av observationen att nedreglering av ChREBP eller TXNIP i INS-1 832/13 celler odlade vid hög glukoshalt ledde till en kraftigt minskad glukosinducerad ökning av VDAC1. Uttryck av VDAC1 kan också nedregleras farmakologiskt av substansen resveratrol som utvinns ur grapefruktskal, vilket motverkade effekten av högt glukos.

Sammanfattningsvis föreslår vi att ett förändrat uttryck av VDAC1 och VDAC2, eventuellt via ökade intracellulära stresssignaler, är en nyckelkomponent i patogenesen av glucotoxicitet vilket leder till strukturella och funktionella förändringar i pankreatiska insulin-producerande celler som resulterar i försämrad insulinutsöndring.

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