From the pancreatic beta cell to the endothelium:Pathophysiological aspects of Type 2 Diabetes

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From the pancreatic β-cell to the endothelium: *Pathophysiological aspects of Type 2 Diabetes*

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Abstract

The global increase in incidence of Diabetes Mellitus has assumed epidemic proportions. Type 2 Diabetes is the most prevalent form of diabetes, comprising 90% of the patients. In Type 2 Diabetes, two fundamental processes con-tribute to the development of the disease: insufficient insulin secretion from the pancreatic β-cell and insulin resistance in the target organs. This leads to loss of control of blood glucose levels, the hallmark of all forms of diabetes. Al-though blood glucose levels can be controlled by a variety of life-style and pharmacological interventions, complications often arise. These complications include cardiovascular disease, retinopathy, neuropathy, and nephropathy. In this thesis, different aspects of pathophysiological mechanisms in Type 2 Diabetes were studied. The aims were (i) to identify the voltage-gated calcium channel that is coupled to glucose-stimulated insulin secretion in the rat clonal β-cell line INS-1 832/13; (ii) to investigate the mechanism of β-cell adaptation in the C57BL/6J mouse model of insulin resistance; (iii) to determine whether glucose tolerance is a feature in the RIP2-Cre mouse model, which is commonly used for β-cell specific knockout of genes; and (iv) to study the presence of insulin receptors and IGF-I receptors in human endothelial cells of different origin. It was established that CaV1.2 is the main voltage-gated calcium channel coupled to glucose-stimulated insulin secretion in INS-1 832/13 cells, con-firming previous results obtained from mouse β-cells. C57BL/6J mice on a high-fat diet become insulin resistant but do not develop diabetes. The adaptation could be attributed to compensatory hypersecretion of insulin, which may be due to a shift in utilization of metabolic fuels from glucose to fatty acids and amino acids. This is reflected by increased mitochondrial mass observed in the β-cells of the insulin-resistant C57BL/6J mice. C57BL/6J mice were also used for backcrossing RIP2-Cre mice onto a pure genetic background. The expression of Cre recombinase did not affect glucose tolerance, insulin secretion or β-cell mass in this pure genetic background. Therefore, this mouse line can be used in β-cell specific knockout studies, where there is a focus on glucose homeostasis. Human endothelial cells from coronary artery and umbilical vein expressed more IGF-I receptors than insulin receptors. Indications of the presence of insulin/IGF-I hybrid receptors were found in both endothelial cell types. These results reflect the importance of IGF-I in the development of vascular complications of Diabetes Mellitus. Thus, our work has examined aspects of the pathogenic mechanisms involved in perturbations of both secretion and action of insulin, highlighting the complexity of Type 2 Diabetes.

Key words: β-cells, insulin secretion, glucose tolerance, insulin receptors, IGF-I receptors, endothelial cells, Type 2 Diabetes

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Table of Contents

Abstract

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III Fex, M, Wierup, N, Nitert, MD, Ristow, M, Mulder, H (2007). *RIP2-Cre mice bred onto a pure C57BL/6J background exhibit unaltered glucose tolerance.* Journal of Endocrinology 194 (3): 551-5.

IV Nitert, MD, Chisalita, SI, Olsson, K, Bornfeldt, KE, Arnqvist, HJ (2005). *IGF-I/Insulin hybrid receptors in human endothelial cells.* Molecular and Cellular Endocrinology 229 (1-2): 31-7.

V Chisalita, SI, Nitert, MD, Arnqvist, HJ (2006). *Characterisation of receptors for IGF-I and insulin; evidence for hybrid insulin/IGF-I receptor in human coronary artery endothelial cells.* Growth Hormone & IGF Research 16 (4): 258-66.

Abbreviations

ATP	adenosine triphosphate
BCH	2-amino-2-norbornanecarboxylic acid
$[Ca^{2+}]_i$	intracellular calcium concentration
Ca_V	voltage-gated calcium channel
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
CoA	coenzyme A
COXIV	cytochrome c oxidase IV
CPT-I	carnitine palmitoyl transferase I
ERK	extracellular signal-regulated kinase
FAD	flavine adenine dinucleotide
GK	Goto-Kakizaki
HCAEC	human coronary artery endothelial cells
HF	high fat
HUVEC	human umbilical vein endothelial cells
IC_{50} value	the concentration needed for half-maximal displacement of the radioactively labeled substrate
IGF-I	insulin-like growth factor I
IGFBP	insulin-like growth factor I binding protein
IRS	insulin receptor substrate
IVGTT	intravenous glucose tolerance test
K_{ATP}-channel	ATP-sensitive potassium channel
MAPK	mitogen-activated protein kinase
mtDNA	mitochondrial DNA
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
Nnt	nicotinamide nucleotide transhydrogenase
NO	nitric oxide
NRF-1	nuclear respiratory factor 1
OLETF rat	Otsuka Long Evans Tokushima Fatty rat
PGC-1	peroxisome proliferator-activated receptor γ coactivator-1
PI-3K	phosphatidylinositol 3 kinase
PKB	protein kinase B
RIP2-Cre	rat insulin promoter 2-Cre recombinase
RNAi	RNA interference
siRNA	short interfering RNA
TFAM	mitochondrial transcription factor A
UCP2	uncoupling protein 2

Introduction

Diabetes Mellitus

Background

Diabetes Mellitus is characterized by increased blood glucose levels. The incidence of diabetes is on the rise globally [1]. A recent study predicted an increase in the prevalence of diabetes in the United States to 14.5% of the population, or 37.7 million people, by 2031 [2]. The World Health Organisation predicts that 300 million people will suffer from diabetes in 2025, a devastating rise from the 137 million in 1997 [3, 4]. The largest increase will occur in the developing world, particularly in Asia, due to profound changes in demographics, epidemiology and socioeconomical conditions [5]. China and India, which already now have the largest number of patients with diabetes, will be afflicted by the greatest numbers of new diagnoses [4]. In the same study, the number of people suffering from diabetes in Sweden is predicted to be 827 000 people, or 11.2% of the population in 2025.

Types of Diabetes Mellitus

Diabetes Mellitus is not a single disease but a group of metabolic diseases, all of which exhibit the hallmark increase in blood glucose levels. Traditionally, Diabetes Mellitus has been subdivided in two types. Type 1 Diabetes, or Juvenile Diabetes, is usually diagnosed in childhood or adolescence, and is caused by an absolute deficiency in insulin secretion. This type of diabetes results from an autoimmune attack on the pancreatic β-cells, which leads to their destruction. Insulin can no longer be produced and control over blood glucose levels is lost. At the time of diagnosis, patients have lost a large proportion of their β-cells, rendering them unable to control blood glucose levels by secreting insulin. This type of diabetes is treated with exogenous insulin.

Type 2 Diabetes manifests itself typically later in life and is associated with aging, obesity, lack of exercise, and insulin resistance in adipose tissue, liver and muscle [6, 7]. Approximately 90% of all patients with Diabetes suffer from Type 2 Diabetes [1]. Alarmingly, the diagnosis of this form of Diabetes is now becoming more frequent in younger individuals. Type 2 diabetes is characterized by the presence of two basic abnormalities: impaired insulin secretion and decreased insulin sensitivity. The pathophysiological spectrum of Type 2 Diabetes spans from insulin resistance with a minor secretory defect to a predominant secretory defect with near-normal insulin sensitivity [8]. Thus, blood glucose levels may increase despite high levels of circulating insulin, as a direct consequence of insulin resistance. In Type 2 Diabetes, secretory defects in β-cells are frequently present before overt diabetes sets in [9, 10].

Genes as well as environmental conditions play a role in the pathogenesis of Type 2 Diabetes. Patients generally have alterations in various genes, each having a partial and additive effect. Genetic susceptibility is made up of different genes and gene combinations [11]. Gene mapping has identified more than 30 chromosomal regions each containing one or more susceptibility genes [11]: the penetration rate of these genetic regions varies in different ethnic groups [8]. The field of genetics has undergone a paradigm shift in the last year. Genome-wide scans, which are performed in large population samples and are not hypothesis-based, have yielded major results. The genes that have

been identified as associated with Type 2 Diabetes in multiple population samples in these scans are *TCF7L2, IGF2BP2, CDKAL1, HHEX, CDKN2B, KCJN11, PPARG* [12-16]. The transcription factor TCF7L2 has been shown to be more strongly associated with Type 2 Diabetes than any other gene previously identified [17]. Moreover, the majority of diabetes genes appears to be implicated in β-cell function. Activation of the genetic predisposition requires the presence of environmental and behavioral factors, especially those associated with lifestyle. Risk factors for Type 2 Diabetes can be classified as non-modifiable and modifiable. Non-modifiable risk factors include genetic factors, age and gender, and previous gestational diabetes. Modifiable risk factors are obesity, with a focus on the distribution of adipose tissue, physical inactivity and nutritional factors [18].

Treatment of Type 2 Diabetes is determined by the severity of the symptoms. It ranges from life-style interventions, such as change of diet and exercise habits, to oral medications to increase the output of insulin from the β-cells with sulfonylureas. The sensitivity to insulin can be increased in the liver with metformin, which reduces hepatic glucose production. Thiazolidinediones are used to increase peripheral tissue uptake of insulin [19]. When these treatments fail, one has to resort to treatment with exogenous insulin.

More recently, additional variants of Diabetes Mellitus have been elucidated, blurring the black/white distinction between Type 1 and Type 2 Diabetes. These forms include maturity-onset diabetes of the young (MODY), which results from mutations in single genes, and latent autoimmune diabetes in adults (LADA), which is characterized by clinical features resembling those of Type 2 Diabetes in combination with circulating auto-antibodies in adults.

Complications of Diabetes Mellitus

Diabetes Mellitus is associated with increased risk of vascular complications. Vascular complications can be subdivided in microvascular and macrovascular disease, which is based on the size of the affected arteries. In microvascular disease, the vascular wall thickens and also weakens, leading to protein leakage, bleeding and diminished blood flow through the vessel. Damage of surrounding cells leads to retinopathy, nephropathy and also neuropathy. Microvascular complications, which rarely are isolated occurrences, constitute the major complication in Type 1 Diabetes. In Type 2 Diabetes, microvascular complications are frequently present at diagnosis, with 20% of the patients suffering from retinopathy, 9% neuropathy, and up to 10% exhibiting overt nephropathy [20]. Diabetic nephropathy accounts for approximately 40% of new cases of end-stage renal disease [20]. Peripheral neuropathy and peripheral vascular disease are major risk factors for diabetic foot ulcers and amputations, and are thereby major causes of morbidity in diabetic patients. Diabetic retinopathy, which virtually all patients with diabetes develop within 20 years of diagnosis, is the leading cause of blindness in the adult population [20]. Prolonged hyperglycemia is thought to be the major mechanism of microvascular disease. Other factors, such as insulin resistance, increased body mass index, age of diagnosis and hypertension are also of importance [21-23]. The risk of developing microvascular complications declines when good glycemic control is achieved [24-26].

Atherosclerosis is a chronic inflammatory condition, which is initiated in the endothelium after an injury occurs. The sustained inflammation results from interactions between modified lipoproteins, macrophages and constitu-

ents of the arterial wall. In Type 2 Diabetes, the risk for macrovascular disease is substantially increased [27]. Just as atherosclerosis in non-diabetic patients, macrovascular disease is complicated by thromboembolic disease; deposits of fat and blood clots build up in the vessels, sticking to the vessel walls and thereby hindering blood flow. The clots can detach and block blood flow in slightly smaller vessels. Coronary disease, cerebrovascular disease and peripheral vascular disease are atherosclerotic manifestations of macrovascular disease. The mortality rate from cardiovascular disease is higher in patients with diabetes than in the general population [28]. The risk of developing coronary artery diseases is increased two to four fold in diabetes and the risk of stroke is also amplified. In fact, coronary heart disease is the leading cause of death in Type 2 Diabetes [29]. Indeed, a recent study reported that the risk of cardiovascular mortality is increased in subjects with impaired fasting glucose and impaired glucose tolerance as well as Type 2 Diabetes [29]. Furthermore, the incidence and severity of peripheral artery disease are increased in diabetes [30]. In macrovascular disease, the association between glycemic control and the development of macrovascular complications is not clear [24-26]. Hyperglycemia adds to the effects of oxidative stress and dyslipidemia, which have been suggested to accelerate and exacerbate the inflammatory processes leading to atherosclerosis [31, 32]. Furthermore, diabetes is often accompanied by hypertension, which in itself is a contributing factor to the pathogenesis of heart failure. Also, diabetic cardiomyopathy, which is secondary to diabetes but independent from the presence of coronary atherosclerosis, is a risk factor in heart failure [28]. Increases in circulatory glucose levels result in increased oxidative stress, increased nonenzymatic glycosylation of proteins and cardioneuropathy [33].

Insulin Secretion

Islets of Langerhans

The islets of Langerhans constitute the endocrine pancreas and are spread throughout the pancreas; they are more numerous in the tail region. In humans, the one to two million islets make up only one to two percent of the pancreatic mass. The islets consist of two major cell types. The β-cells produce insulin and constitute 60 to 80% of the islet cell population. The α-cells synthesize glucagon and make up 10 to 30% of the population. The remainder of the cells in the islets is δ-cells, accounting for approximately 5% of the islet cells, and are the source of somatostatin, and PP-cells producing pancreatic polypeptide. In the pancreas, somatostatin inhibits the secretion of insulin and glucagon as well as secretion from the exocrine pancreas. Pancreatic polypeptide decreases secretion from the exocrine pancreas. Generally, the β-cells are located centrally in the islets of humans and rodents, whereas the α-cells and the δ-cells are at the periphery of the islet. β-cells are inter-connected by gap junctions permitting electrical conductivity between the cells. These gap junctions may therefore couple a number of β-cells in a functional unit for insulin secretion. β, α and δ are electrically excitable cells. The islets are innervated by autonomic afferent nerves and are well vascularized. The efferent vessels from the islets drain into the portal vein, exposing the liver to very high concentrations of insulin [34, 35].

Glucose-stimulated insulin secretion

The β-cells in the islets of Langerhans secrete insulin in response to increases in the blood glucose levels. β-cells are uniquely equipped for aerobic metabolism, *i.e.* the conversion of glucose carbons to carbon dioxide and water, which occurs with an efficiency of 80% in β-cells [36]. This probably results from low expression levels of lactate dehydrogenase [36] and plasma membrane lactate/monocarboxylate transporter, while levels of mitochondrial glycerol phosphate dehydrogenase are high [37]. Under these circumstances, the rate of glycolysis will be high, and its end-product pyruvate, will be fully oxidized in the citric acid cycle.

Glucose enters the β-cell through a high-capacity, low-affinity, non-insulin dependent glucose transporter, Glut2 in rodents, which ensures the continuous flow of glucose into the β-cell. It is phosphorylated to glucose-6-phosphate by glucokinase, which has a high K_m and therefore determines the rate of glycolysis. Glycolysis is the enzymatic breakdown of glucose by way of phosphate derivatives with the production of pyruvate and energy stored in high-energy anhydride bonds of ATP. Each glucose molecule gives rise to two pyruvate molecules, two ATP molecules and 2 NADH molecules, the reduced form of nicotinamide adenine dinucleotide. Acetyl-coenzyme A (acetyl-CoA), a two carbon-molecule, is then formed from pyruvate in the pyruvate dehydrogenase complex reducing another NAD^+ to NADH. The acetyl-CoA enters the citric acid cycle in the mitochondria, where the two carbon atoms are oxidized. This process gives rise to more reduced electron carriers, 3NADH and 1$FADH_2$ per acetyl-CoA, as well as the production of 1 ATP.

The reduced electron carriers (NADH and $FADH_2$) are reoxidized in the electron transport chain, a process that culminates in ATP synthesis. The mitochondrial electron transport chain is a multiprotein unit grouped into four complexes (I to IV), which are located within the mitochondrial inner membrane. The final fate of the electrons is reduction of oxygen to water. Complex I, III and IV are reduction- and oxidation-driven proton pumps that use energy carried by the electrons to pump protons out of the matrix. This creates a proton electrochemical potential gradient across the inner mitochondrial membrane. The protons subsequently reenter the mitochondrial matrix via ATP synthase (complex V), and the energy released from the electrochemical gradient drives synthesis of ATP from ADP. For each NADH, a maximum of 3 ATP molecules can be generated whereas 2 ATPs are produced per $FADH_2$ oxidized.

The generated ATP is transported from the mitochondria to the cytoplasm in exchange for ADP by the adenine nucleotide translocator, changing the ratio of ATP to ADP in the cytoplasm. This change in the ratio is thought to close the ATP-sensitive potassium channel (K_{ATP}-channel) resulting in depolarization of the plasma membrane. Subsequently, voltage-sensitive calcium channels open and calcium flows into the cell. Intracellular calcium concentrations in the cytoplasm increase and this triggers the secretion of insulin granules (see figure 1). This constitutes the first phase of insulin secretion, which lasts five to ten minutes [38] (see Figure 1). The second, more sustained, phase of insulin secretion does not involve further closure of K_{ATP}-channels nor changes in total cytoplasmic Ca^{2+} concentrations. Therefore other signaling events as well as local changes in cytoplasmic Ca^{2+} are thought to underlie this phase of insulin secretion.

In the second phase of insulin secretion, the pace of secretion is slower than in the first phase. It has been suggested that the insulin granules secreted

in the first and second phase of insulin secretion originate from different pools of insulin granules [39]. The granules that are secreted during the first phase stem from the readily releasable pool, a small pool of insulin granules that are primed and docked, residing near the plasma membrane [40]. Only approximately 50 granules per cell are secreted during the first phase [40]. The granules secreted during the second phase are thought to belong to a different pool of granules, the reserve pool, and are localized in the cytoplasm further from the plasma membrane. The granules from the reserve pool need to be mobilized and primed before secretion; this is a time-, Ca^{2+}- and ATP-dependent process [41].

The K_{ATP}-channel-/calcium-dependent pathway of insulin secretion is also called the triggering pathway. This is contrasted with the amplifying pathway, which augments insulin secretion that has been provoked by the triggering pathway [42]. The amplifying pathway can be unmasked by conditions where a high concentration of K^+ and the K_{ATP}-channel opener diazoxide, are present; high [K^+] ensures that the plasma membrane is depolarized, and the presence of diazoxide prevents closure of the K_{ATP}-channel. Under these conditions high glucose levels will still enhance insulin secretion despite the fact that the K_{ATP}-channel is bypassed, alas K_{ATP}-independent glucose sensing. Alternatively, when the K_{ATP}-channel is closed, by sulfonylureas, a stimulatory effect of glucose on insulin secretion is still evident.

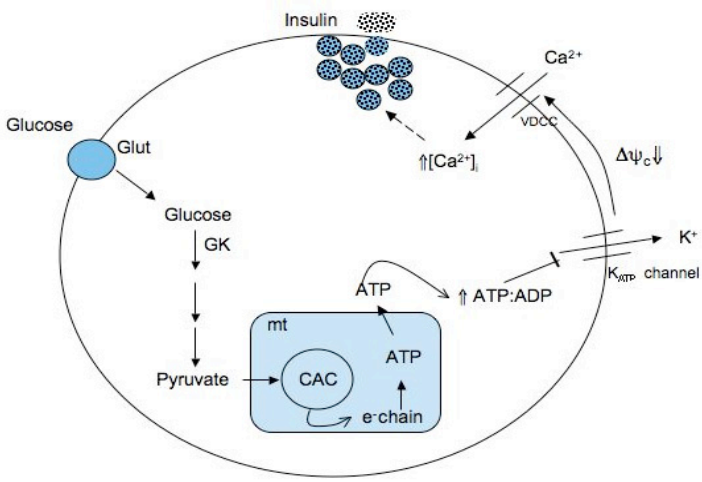

Figure 1. Glucose-stimulated insulin secretion. Glucose enters the cell using the glucose transporter (Glut) and is metabolized in glycolysis, yielding pyruvate. Pyruvate enters the citric acid cycle (CAC) in the mitochondrion (mt). This promotes ATP production by the electron transport chain (e^- chain). ATP is transported from the mitochondrion, increases the ATP:ADP ratio, and closes the K_{ATP} channel. The membrane potential decreases and this opens voltage-gated calcium channels (VDCC) allowing calcium (Ca^{2+}) to enter the cell and signal for the release of insulin granules.

The amplifying pathway does not increase Ca^{2+} concentrations further and relies on the triggering pathway to increase the Ca^{2+} concentrations to permissive levels before it can augment insulin secretion.

The amplifying pathway was first identified in rodents in 1992 [43, 44] and in humans in 1998 [45], and requires prior activation of insulin secretion via the triggering pathway. It is, as yet, unclear what exactly constitutes the amplifying signal, although metabolism of glucose appears to be required for the signal. Mitochondria have been implicated as the main source of the amplifying signal. The additive signal has alternatively been proposed to be ATP, the ATP/ADP ratio, GTP, cAMP, NADPH as well as metabolites that do not directly derive from mitochondria, such as glutamate and malonyl-CoA (see for review [46]). However, for a number of these, e.g., cAMP/protein kinase A, protein kinase C, long-chain acyl-coAs, phospholipase A2, and nitric oxide (NO)/cGMP, there is little supporting evidence [47]. Nevertheless, abundance of ATP is a requirement for the priming of insulin granules from the reserve pool and remains a candidate for being a factor in the amplifying pathway of insulin secretion [40].

Anaplerosis has been suggested to be the source of coupling factors for the amplifying pathway. This could potentially be through the generation of intermediates that stimulate the amplifying pathway or through the generation of reducing equivalents, ATP or GTP. Anaplerosis, *i.e.* the filling up the citric acid cycle with intermediates that are channeled into anabolic pathways, can be executed by conversion of pyruvate into oxaloacetate by pyruvate carboxylase instead of pyruvate into acetyl-CoA by pyruvate dehydrogenase [48] (see Figure 2). Indeed, pyruvate carboxylase is present in high amounts in pancreatic β cells as compared to other islet cells [36, 48]; approximately 40% of pyruvate entering the citric acid cycle enters by carboxylation to oxaloacetate by pyruvate carboxylase [49]. Stimulated flow of intermediates from the citric acid cycle (cataplerosis) into the synthesis of coupling factors or macromolecules is believed to sustain stimulus-secretion coupling and β-cell growth [36]. Also, anaplerosis may be crucial for augmenting the pyruvate/malate shuttle, which results in the formation of cytosolic NADPH [50]. Thus, oxaloacetate, formed in the reaction catalyzed by pyruvate carboxylase, is a substrate for the pyruvate/malate shuttle yielding cytosolic NADPH, which is a putative coupling factor [50].

In addition to the initiator effects of the fuel secretagogues, potentiator molecules exist which enhance secretion at permissive but submaximal fuel secretagogue concentrations. These potentiator molecules include acetylcholine, glucagon-like peptide, gastric inhibitory peptide, and pituitary adenylate cyclase-activating polypeptide [51]. Most of the potentiator molecules employ cAMP or Ca^{2+} as second messenger molecules.

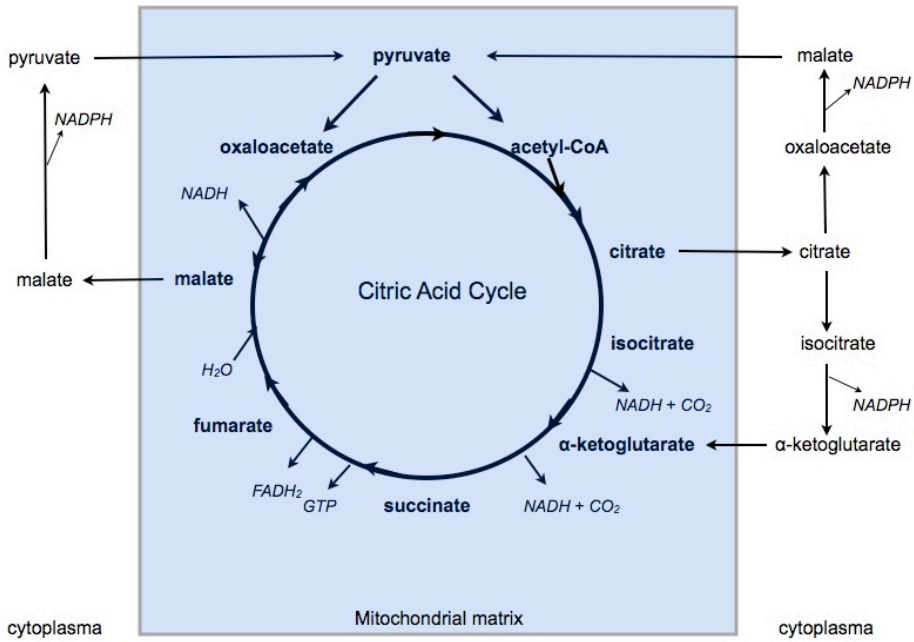

Figure 2. The citric acid cycle in mitochondria. Pyruvate enters the mitochondria and is converted into acetyl-CoA or oxaloacetate and enters the cycle. The cycle produces NADH, $FADH_2$, GTP and CO_2. NADH and $FADH_2$ subsequently donate electrons to the electron transport chain, which is coupled to the synthesis of ATP.

The role of mitochondria in β-cells

Mitochondria may have originally been derived from the symbiotic association of oxidative bacteria and glycolytic pro-eukaryotic cells [52]. The main function of the mitochondria is the generation of energy in the form of ATP. The number of mitochondria per eukaryotic cell varies from several hundred to several thousand, depending on the cell type [53]. However, a study using targeted green fluorescent proteins indicated that mitochondria constitute a continuous network in the cell instead of individual units [54]. Each mitochondrion possesses a few copies of maternally-inherited mitochondrial DNA (mtDNA), which is circular and consists of coding sequences only. The proofreading capacity of mitochondrial DNA polymerase is poor and the mutation rate in mtDNA is therefore high. The mtDNA is located close to the respiratory chain and is therefore sensitive to oxidative stress. However, since a few copies of mtDNA are present in each mitochondrion, mutations are generally present in only a fraction of the mtDNA in the cell giving rise to heteroplasmy [53]. Human mtDNA encodes for tRNAs and 13 polypeptides that encode for subunits of the multi-subunit enzyme complexes for respiration. However, the majority of the subunits for these complexes is encoded in the nucleus and imported into mitochondria as proteins. Nuclear-encoded proteins, such as mitochondrial transcription factor A (TFAM), also determine the transcriptional rate [55]. Deletion of the Tfam gene from β-cells in mice causes a diabetic phe-

notype with decreased glucose-stimulated insulin secretion [56]. In humans, a maternally inherited mutation of a mitochondrially encoded tRNA gives rise to diabetes; mutations in mtDNA giving rise to diabetes, account for approximately 1% of all diabetes cases [57]. This type of diabetes is not associated with insulin resistance but rather with reduced glucose-stimulated insulin secretion [58].

Knock-down of nicotinamide nucleotide transhydrogenase (Nnt), which is a nuclear-encoded mitochondrial protein in mouse β-cells produces a dramatic reduction in insulin secretion and prevents the rise in intracellular Ca^{2+} after stimulation with glucose [59]. This is the result of impaired ATP production in response to glucose, which subsequently fails to close the K_{ATP}-channel [59]. Similar results are observed in C57BL/6J mice in which Nnt protein is deleted [60]. Nnt is located in the inner mitochondrial membrane and it serves as a redox-driven proton pump catalyzing the reversible reduction of NADP to NADPH by NADH, which is converted into NAD^+, pumping a hydrogen atom from the cytosol to the mitochondrial matrix [61]. Nnt is a major generator of NADPH and it couples this production to the rate of mitochondrial metabolism. It also couples NADPH production to the production of reactive oxygen species generated by the electron transport chain [61]. Reactive oxygen species stimulate the activity of uncoupling protein-2 (UCP2), leading to increased proton leakage across the inner mitochondrial membrane, reducing the electromotive force and thereby ATP synthesis [62]. Nnt may provide a protective buffer against the dissipation of either the cellular redox power or of the mitochondrial energy supply [61].

Lipids in insulin secretion

Insulin secretion is influenced by the presence of lipids under both normal and pathophysiological circumstances [63-66]. Mitochondrial ATP production can also result from oxidation by fatty acids; long-chain acyl-CoAs are imported into the mitochondria for oxidation, a process regulated by carnitine palmitoyl transferase I (CPT-I). However, oxidation by fatty acids alone cannot provoke insulin secretion. This conundrum is unresolved but may relate to the fact that the acetyl-CoA produced by fatty acid oxidation in mitochondria does not provide a net addition of carbons to the citric acid cycle [67]. Under such circumstances, anaplerosis and cataplerosis, processes critical for glucose-stimulated insulin secretion are not stimulated.

It has been suggested that in glucose-stimulated β-cells, citrate from the citric acid cycle is exported from the mitochondria. In the cytosol, citrate is hydrolyzed, and the carbons are transferred to CoA to form acetyl-CoA. Acetyl-CoA carboxylase catalyzes the synthesis of malonyl-CoA, which is a lipid precursor. Malonyl-CoA prevents fatty acid transport into the mitochondria by inhibition of CPT-I [68]. Thus, fatty acid oxidation is reduced, favoring the synthesis of long-chain acyl-CoAs in the cytosol (for a review see [69]). Long-chain acyl-CoA is thought to be an effector molecule, which may affect multiple targets in the β-cell, e.g., stimulation of CPT-I in the absence of stimulatory glucose concentrations [70], and activation of protein kinase C [71]. Long-chain acyl-CoA is thought to stimulate endoplasmatic reticulum Ca^{2+} ATPase, and peroxisome proliferation [69].

Under non-stimulatory glucose conditions, endogenous triacylglycerols are important fuels in intermediary metabolism of pancreatic islets. Free fatty acids potentiate glucose-stimulated insulin secretion both *in vivo* and *in vitro* [63, 64, 72]. Whether the source of fatty acids for this potentiation is intracellu-

lar or extracellular is unclear. On one hand, β-cells store triacylglycerols and express the hormone-sensitive lipase, which may generate lipids for stimulus-secretion coupling [73, 74]. On the other hand, it has been speculated that the effects of fatty acids on β-cells may, at least in part, be mediated by G-protein coupled transmembrane receptors, GPR-40, without entering the β-cell [75].

Free fatty acids may exert different effects on insulin secretion depending on the duration of exposure. Short-term exposure, *e.g.* 3 hours or less, increases glucose-stimulated insulin secretion whereas long-term exposure, *e.g.* more than 6 hours, has the opposite effect [72, 76]. Chronic exposure to free fatty acids even decreases glucose-induced proinsulin and total biosynthesis in rat islets [72, 77]. Hyperlipidemia, which is present in obesity and frequently in Type 2 Diabetes patients, increases the cytosolic concentration of long-chain CoAs. Hyperglycemia also increases the cytosolic long-chain acyl-CoA concentration via the inhibition of CPT-I by malonyl-CoA [69]. Both these events may play a role in "glucolipotoxicity" [78].

Calcium and voltage-gated calcium channels

Voltage-gated calcium channels are part of a family of multi-subunit ion channels. The channels play a key role in triggering Ca^{2+} signaling in a wide variety of cells. Their function is to serve as Ca^{2+}-conducting pores in the plasma membrane. When the plasma membrane depolarizes, the channel pores undergo a rapid conformational switch, from the impermeable state to the permeable, allowing the influx of extracellular Ca^{2+} into the cytoplasm [79]. The channels consist of an α_1, an $\alpha_2\delta$, and a β-subunit. The association of the α subunit with the other subunits is required for the functional expression of the channels.

The α_1 subunit has different primary structures and mainly confers the electrophysiological and pharmacological properties of the channel. The α_1-subunits form the ion-conducting pore of the calcium channel. Several pore-forming calcium channel α_1-subunits of voltage-sensitive calcium channels have been identified in rat pancreatic islets and β-cell lines; $Ca_V1.2$, $Ca_V1.3$, $Ca_V2.1$, $Ca_V2.2$, $Ca_V2.3$, and $Ca_V3.1$ [80-86]. The contributions of the different calcium channels to the increase in $[Ca^{2+}]_i$ have been extensively studied. Approximately 60 to 70% of the rat and mouse whole-cell current in β-cells are resistant to dihydropyridines [83, 87-92]. The major dihydropyridine-sensitive calcium channels in rat β-cells are $Ca_V1.2$ or $Ca_V1.3$, with different studies emphasizing the role of either $Ca_V1.2$ or $Ca_V1.3$ [90, 93, 94]. In mouse β-cells, $Ca_V1.2$ is the only major dihydropyridine-sensitive calcium channel [88], although $Ca_V1.3$ has also been reported to be present [91, 95]. However, $Ca_V1.3$ has been found not to affect insulin secretion in a mouse knock-out model and probably plays a minor role in insulin secretion in mouse β-cells [96]. In human islets, both $Ca_V1.2$ and $Ca_V1.3$ are present [89]. Calcium currents that can be inhibited by ω-conotoxin GVIA, which is known to block $Ca_V2.2$, have been reported in rat β-cells but the role of this calcium channel in insulin secretion is controversial [97, 98]. $Ca_V2.3$, which is inhibited by SNX-482, accounts for another 18% of the Ca^{2+} current in mouse β-cells [99]. The role of $Ca_V2.3$ in rat β-cells is not clear since it has been reported that 25% of insulin secretion in rat INS-1 β-cells was inhibited by SNX-482 [100], whereas others reported no effect of SNX-482 in the same cell line [101]. In human islets, $Ca_V2.3$ has been identified immunohistochemically [81]. Low-voltage activated calcium channels; $Ca_V3.1$, $Ca_V3.2$ and $Ca_V3.3$, are not found in mouse pancreatic β-cells

[102]. In humans, however, these low-voltage calcium channels are readily detected [103, 104].

The β subunit of the voltage-gated calcium channels is located in the cytoplasm and is linked to the α_1 subunit [105]. The α_2 subunit is bound by a disulfide bridge to its post-translationally cleaved transmembrane δ peptide that links it to the α_1 subunit [106]. Both $\alpha_2\delta$ and β subunits modulate the channel. The limited mobility of the N-terminal part of the α_1 subunit appears important for the integration of the β subunit and the C-terminus of the α_1 subunit in inactivation of the channel in the case of $Ca_V1.2$ [107].

The electrical activity of the pancreatic β-cell consists of oscillations in the membrane potential between depolarized plateaus, from which Ca^{2+}-dependent action potentials originate and which are separated by repolarizations of the membrane potential [39]. The Ca^{2+} influx into the cell is pulsatile and has been coupled to oscillatory insulin secretion [108-111]. In β-cells, Ca^{2+} microdomains, *i.e.* small domains with a local high Ca^{2+} concentration, are presumed to exist beneath the plasma membrane (reviewed in [112]). The Ca^{2+} concentration in these domains is high enough to stimulate insulin vesicle release as opposed to the lower Ca^{2+} concentration in the bulk of the cytosol. The intracellular calcium store in the endoplasmatic reticulum may also play a role in the local variations in Ca^{2+} influx [113, 114] by prompting local variations in membrane potential when the endoplasmatic reticulum calcium is depleted. The endoplasmatic reticulum calcium store is filled when calcium enters the cells after stimulation with secretagogues [113]. In β-cells, co-localization of $Ca_V1.2$ and insulin granules has been identified [115, 116]. This would then create a Ca^{2+} microdomain near the insulin granules initiating granule release. Voltage-gated calcium channels that are not sensitive to dihydropyridines appear not to be co-localized with granules and apparently do not play a major role in triggering insulin granule release [88]. It has been proposed that these channels instead exert control of electrical activity [117], and in the case of $Ca_V2.3$, are more important for the second phase of insulin secretion [99]. Another Ca^{2+} microdomain may be formed in the immediate surroundings of a proportion of the insulin granule pool located close to the plasma membrane but not surrounding insulin granules further from the plasma membrane [118, 119].

A number of studies have linked perturbations in expression of voltage-gated calcium channels with Diabetes Mellitus. Wistar rats treated with streptozocin as well as OLETF rats, which are models for Type 2 Diabetes, exhibit decreased mRNA levels of the $Ca_V1.2$ α_1 subunit but also β_2 and β_3 subunit [120]. These rats show higher basal insulin secretion and profoundly impaired glucose-stimulated insulin secretion. Currents through the $Ca_V1.2$ and/or the $Ca_V1.3$ channels are, however, increased in Goto-Kakizaki (GK) rats and also in Wistar rats treated with streptozocin (at a slightly lower dose) [121, 122]. In humans, a rare mutation in the $Ca_V1.2$ gene has been associated with episodic serum hypoglycemia [123].

Insulin Action

Insulin

Insulin is a hormone that is required for normal growth and development. Its unique function is to directly lower blood glucose levels. In humans, the half-life of insulin is approximately 5 to 10 minutes. Insulin arises from a precursor

that consists of A, B and C domains. The C-domain is cleaved from proinsulin, giving rise to mature insulin, which consists of 51 amino acids, and the connecting (C)-peptide. Insulin mediates its role through binding to its receptor on the membrane of target cells. The binding of insulin to its receptor triggers signals to branching series of intracellular pathways regulating cell metabolism, growth, differentiation and survival [124]. Insulin increases protein synthesis by increasing the number of initiated ribosomes and thereby promotes the initiation of translation.

Target organs

Insulin exerts its action in a large number of tissues. The major targets for insulin, however, are skeletal muscle, adipose tissue and liver. Skeletal muscle and liver are essential for maintaining normal glucose homeostasis. Insulin exerts its effect on the target cells by binding to the insulin receptors (see below). In many target organs, insulin activates the rapid synthesis of glycogen by stimulating glycogen synthase activity. This accounts for approximately 70% of all glucose that is disposed post-prandially [125]. In skeletal muscle, the major effect of insulin is stimulation of the translocation of the Glut4 glucose transporter to the plasma membrane, which ensures the uptake of glucose into the skeletal muscle cell. Seventy to 80% of all insulin-mediated glucose disposal is accounted for by skeletal muscle. In hepatocytes, insulin receptor binding decreases the hepatic glucose production conferred by gluconeogenesis and glycogenolysis, thereby controlling blood glucose concentrations. Insulin stimulates glycolysis and glycogen synthesis in the liver. The insulin concentration in the portal vein is cardinal for conveying these effect of insulin [125]. In contrast to skeletal muscle, insulin only plays a permissive role, not a stimulatory role, in glucose uptake by hepatocytes, which express Glut2.

In adipocytes, insulin binding to its receptor also activates Glut4 translocation to the plasma membrane. However, glucose uptake in adipocytes only accounts for approximately 5% of insulin-mediated glucose uptake [125]. Activation of Glut4 translocation results in an acute 20- to 40-fold stimulation of transportation rates of glucose [126]. If excess glucose is available, insulin stimulates glucose metabolism to glycerol 3-phosphate, which is then coupled to fatty acids to form triacylglycerol. Triacylglycerol accounts for fat storage in adipocytes. Insulin inhibits lipolysis, by inactivation of hormone-sensitive lipase [127]. Pancreatic β-cells, in the rat and the mouse, also express receptors for insulin [128, 129]. These receptors are involved in control of β-cell mass, and may be involved in glucose-stimulated insulin secretion. β-cell-specific knock-out of the insulin receptor results in a decrease in first phase glucose-stimulated insulin secretion and progressive age-dependent glucose intolerance [130].

Insulin resistance

Insulin resistance is a fundamental abnormality in the pathophysiology of Type 2 Diabetes mellitus [131]. Insulin resistance typically occurs before the onset of hyperglycemia [10, 131]. In insulin resistance, glucose metabolism is affected while other effects of insulin may not be as attenuated [33]. Insulin resistance is present in up to 50% of people with essential hypertension and it has been associated with congestive heart failure [132].

Insulin resistance implies a reduced response to insulin in its target tissues. This comprises decreased insulin-stimulated glucose uptake in skeletal

muscle and impaired suppression of endogenous glucose production by the liver, both of which are critical for maintaining normal glucose homeostasis. Insulin resistance is partly explained by genetic factors. It may be the earliest identifier of subsequent cardiovascular disease risk and it is an early diabetes risk factor. Insulin resistance is frequently observed in Type 2 Diabetes, but also in people with central obesity, atherosclerotic cardiovascular disease, and most dyslipidemias. Hyperlipidemia induces insulin resistance and many changes in nutrient and hormonal levels, which all could affect β-cell function as well as skeletal muscle and liver. Not all insulin-resistant individuals, however, develop Type 2 Diabetes. Since insulin action involves a cascade of events, disruptions at any stage of the cascade may lead to insulin resistance.

Adipose tissue is proposed to play a major role in the development of insulin resistance [133]. Adipose tissue affects whole body glucose metabolism by regulating the circulating free fatty acid concentrations. Moreover, adipose tissue has been identified as an endocrine organ, and is able to secrete a number of adipokines, including leptin, adiponectin, tumor necrosis factor-α, interleukin-6, resistin, and insulin-like growth factor I (IGF-I) [66]. Adipokines influence insulin sensitivity and food intake, implicating another pathway for adipose tissue to influence whole body glucose metabolism [134]. Associations between adiponectin, insulin sensitivity, cardiovascular disease and endothelial functions have been reported, linking lipids to the occurrence of vascular complications of diabetes [135]. In addition to regulation of food intake, a functional role of leptin may be the regulation of intracellular homeostasis of fatty acids and triglycerides in non-adipocytes. Hereby, a sufficient supply of fatty acids is maintained for essential cellular functions while triglyceride overload is avoided [133]. In diet-induced obesity, leptin resistance develops and this may be part of the slow overaccumulation of lipids observed in many tissues in Type 2 Diabetes, i.e., lipotoxicity [66]. A widely embraced hypothesis is that insulin resistance is the result of changes in the fat distribution among adipose tissue, muscle, and liver. These changes lead to an increase in intracellular fatty acid metabolites, *e.g.* acyl Co-A and diacylglycerol, in liver and muscle. This in turn may lead to activation of serine kinases, which are capable of phosphorylating serine residues in proteins involved in the insulin receptor signaling pathway, such as insulin receptor substrate (IRS) proteins and phosphoinositol 3-kinase (PI-3K). The serine phosphorylation, in contrast to tyrosine phosphorylation, leads to a decrease in the activity of these proteins and thereby possibly to insulin resistance [136]. One of the consequences of decreased activity of insulin signaling proteins is diminished translocation of the insulin-dependent glucose transporter Glut4 in skeletal muscle cells [137]. Interestingly, a compromised translocation of Glut2 to the plasma membrane of β-cells in C57BL/6J mice fed a high-fat diet has also been observed [138].

Glycogen synthase activity is reduced by 35 to 50% in patients with Type 2 Diabetes [139]. The allosteric activation of glycogen synthase by glucose-6-phosphate is diminished in these patients. This decreases glycogen production in Type 2 Diabetes patients. The mechanism behind this has not been fully elucidated.

Mitochondria are involved in insulin resistance as well. In lean elderly people with severe insulin resistance in muscle and higher levels of triglycerides in muscle and liver, mitochondrial oxidative activity and ATP synthesis are decreased [140]. In fact, the disturbances in mitochondrial fatty acid metabolism probably lead to increases in intracellular fatty acid metabolites, which may precipate insulin resistance as described above.

Insulin resistance is usually accompanied by an adaptive increase in insulin secretion from the β-cells. This adaptation involves expansion of β-cell mass and maintenance of normal glucose responsiveness of the β-cells. In those obese individuals, which progress to overt Type 2 Diabetes, this adaptive process has failed and may contribute to hyperglycemia [141].

Insulin receptor

The insulin receptor belongs to the family of tyrosine kinase receptors: it is heterotetramerical in structure and consists of two heterodimers. The heterodimer is made up of a 130 kDa α-subunit and a 97 kDa β-subunit, which are connected by covalent disulfide bridges. The heterodimers are joined together by disulfide bridges between the two α-subunits. The dimerization of two heterodimers and the disulphide linkage take place in the endoplasmatic reticulum. The final constellation of the receptor is βααβ (see Figure 3). The α-subunit is extracellular and contains the ligand-binding domain, whereas the β-subunit spans the plasma membrane and is mainly located in the cytoplasm. The β-subunit hosts the tyrosine kinase site, which is autophosphorylated upon ligand binding. The tyrosine kinase domain subsequently phosphorylates tyrosine residues in other signaling molecules recruited to the receptor after ligand binding. The autophosphorylation occurring after ligand binding is mainly a trans-autophosphorylation, *i.e.*, ligand will bind to the α-subunit of heterodimer 1 which then results in the autophosphorylation of the β-subunit of heterodimer 2, although cis-autophosphorylation does occur [142-144]. The activation of the tyrosine kinase of the β-subunit of heterodimer 2 by phosphorylation will then result in phosphorylation of the tyrosine kinase of the β-subunit of the first heterodimer. The end result will be activation of tyrosine kinases in both β-subunits of the receptor. This mechanism has been elucidated by elegant studies by Treadway *et al.* [145].

The ligand binding domain of the insulin receptor is reported to be complex and discontinuous. It is made up by the N-terminus, the cysteine-rich domain and the C-terminus of the α-subunit [146]. Ligand binding to the insulin receptor fits the model of negative cooperativity. This means that when each heterodimer is individually studied *in vitro* by reducing the disulfide bridges between the two α-subunits, one high-affinity binding site can be detected in each. However, when two heterodimers form the typical heterotetrameric structure of the insulin receptor, only one high-affinity binding site remains and the affinity of the other binding site decreases considerably. The binding curves on a Scatchard plot are curvilinear which is consistent with the model of negative cooperativity [147]. This model has been confirmed [148], and it was proposed that the association of the α-subunits resulted in a physical proximity of the two potential binding sites which is a requirement for high-affinity insulin binding. The insulin receptor can bind IGF-I although the affinity for IGF-I is 100 to 1000 times lower than for insulin.

The insulin receptor exists in two isoforms. Insulin receptor A does not contain the 36 base pairs of exon 11 encoding the last 12 amino acids of the α-subunit. Insulin receptor isoform B contains these 12 amino acids of exon 11. The alternative splicing of exon 11 appears to be modulated by sequences in intron 10 [149]. The relative abundance of the two isoforms in different cell types and tissues varies and is subject to regulation by factors which are tissue-specific, developmental, and hormonal [150, 151]. Brown pre-adipocytes, HepG2 hepatoblastoma cells, hematopoietic and neuronal cells express only isoform A; placental, kidney, adipose tissue and skeletal muscle express both

forms whereas adult liver, many fetal tissues and cancer cells contain predominantly isoform B [152-154]. Rat pancreas has been shown to contain mainly isoform A [155], whereas rat and mouse pancreatic β-cells have been reported to contain both isoforms [156]. Human islets express both isoforms of the insulin receptor in a ratio of 40% isoform A to 60% isoform B [157], the level of isoform B increased to 80% upon exposure to hyperglycemia for 5 days. Isoform A reportedly is coupled to transcription of the insulin gene, whereas isoform B stimulates that of glucokinase in β-cells [158].

The presence or absence of exon 11 has consequences for the ligand binding affinity of the receptor. Insulin receptor A is reported to bind IGF-II with high affinity [153], whereas insulin receptor B has a higher affinity for insulin [159]. This difference in the affinity for insulin between the isoforms is also reflected in the decreased sensitivity of isoform B for metabolic actions of insulin [160]. The missing 12 amino acids thus appear very important for the high affinity binding of insulin [146]. However, certain side chains contribute differentially to insulin binding in each isoform so that different molecular mechanisms may be used in order to confer affinity [161]. It has been reported that adipose tissue and skeletal muscle in obese subjects have increased levels of isoform B compared with control subjects [162].

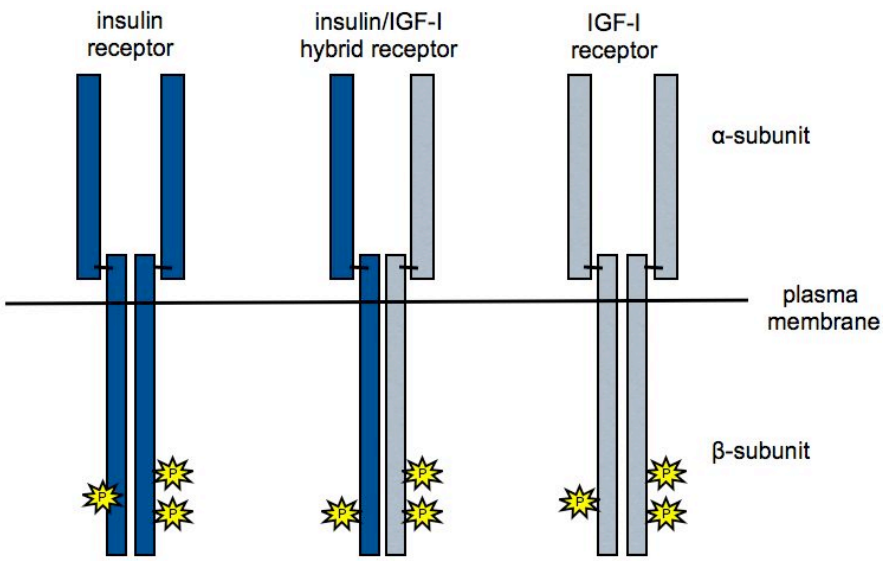

Figure 3. Insulin receptor, IGF-I receptor and insulin/IGF-I hybrid receptor. P is tyrosine phosphorylation site.

Effects of insulin on endothelial cells

In cultured bovine and rabbit microvascular endothelial cells, insulin (at 1-10 nM concentrations), stimulates glucose transport, amino acid transport, glucose oxidation, and DNA synthesis (reviewed in [163]). Insulin does not exert these effects in cultured macrovascular endothelial cells [163]. The hormone

has been reported to affect hemodynamically active substances. Insulin, infused at 0.78 nmol per kg bodyweight, appears to stimulate release of both the vasoconstrictor endothelin-1 and the vasodilator NO but results in no significant hemodynamic effect in human forearm venous circulation [164]. In human umbilical vein endothelial cells (HUVEC) and bovine aortic endothelial cells, supraphysiological concentrations (1-10 μM) of insulin stimulated NO production [165, 166]. High insulin concentrations (0.08 to 4 nM), such as those in hyperinsulinemia, exacerbate neutrophile adhesion to endothelial cells [167]. These concentrations stimulate endothelial intercellular adhesion molecule-1 expression through activation of protein kinase C (PKC) and mitogen-activated protein kinase (MAPK), making the vascular wall more prone to atherosclerotic development [167]. Early growth response gene is a key transcription factor involved in vascular pathophysiology. In murine glomerular vascular endothelial cells, insulin increases expression of this transcription factor through activation of ERK1/2 activation [168]. Insulin exerts anti-apoptotic effects on endothelial cells via phosphatidylinositol 3-kinase (PI-3K) and protein kinase B (PKB) downstream [169]. However, the insulin concentrations used in these studies were high, 100 nM, and an effect of insulin on the IGF-I receptor cannot be excluded.

Ligand binding studies have identified the presence of insulin receptors on human umbilical vein and arterial endothelial cells [170, 171]. HUVEC contain insulin receptor protein, which reportedly can be phosphorylated by stimulation with the relatively high concentration of 100 nM insulin [172].

IGF-I, the IGF-I receptor, and hybrid receptors

Insulin-like growth factor I (IGF-I)

IGF-I is a protein consisting of 70 amino acids and a molecular weight of 7650 Dalton [173]. IGF-I is mainly produced by hepatocytes in response to stimulation of growth hormone receptors [174]. Growth hormone also stimulates IGF-I expression in pancreas, muscle, intestine, kidney, brain, and adipose tissue [175]; expression of IGF-I can also be induced by other factors. IGF-I is subsequently secreted into the circulation. Peripheral tissues can also produce IGF-I, which then acts in an autocrine and paracrine fashion. Circulating IGF-I levels are high during fetal growth, decrease postnatally, and increase again in puberty, after which they decline throughout adult life [174]. Free and potentially active IGF-I accounts for less than 1% of the circulating IGF-I. IGF-I in the circulation is mainly transported in a complex with IGF-I binding protein (IGFBP) 3 and a carrier protein, acid-labile subunit. IGFBP-3 is one of six human IGFBPs, all of which increase the half-life of IGF-I and regulate its biological activity [176]. The IGFBPs are expressed differentially in different tissues. Previous studies have identified mRNA for IGFBP 2 to 6 in bovine aortic endothelial cells [177, 178]. In pulmonary artery endothelial cells, mRNA for the IGFBPs 3, 4 and 6 was identified [177]. IGFBPs can be proteolytically cleaved, releasing IGF-I [179].

IGFI is a fetal and postnatal somatic growth factor. It has been proposed that the endocrine component of IGF-I-stimulated growth is dependent on growth hormone whereas the autocrine/paracrine component is independent of growth hormone [180]. IGF-I can stimulate DNA, RNA and protein synthesis. It can protect cells from apoptosis; it is involved in cellular differentiation

[181]; and it is important for the maintenance of normal cardiovascular structure and function during adult life [182, 183]. Systemic increases in IGF-I concentrations have been found to increase insulin sensitivity [184].

IGF-I is also critical for normal vascular development in the retina [185]. IGF-I increases NO production and thereby stimulates vasodilation in HUVEC [165, 172]. It was found to stimulate ERK 1/2 and ERK 5 phosphorylation in porcine aortic endothelial cells [186]. ERK5 is involved in the response to oxidative stress and shear stress. IGF-I also stimulates phosphorylation of PKB in these cells independent of ERK phosphorylation. Both these pathways lead to activation of NF-κB, which is involved in apoptosis and the inflammatory response. The activation of the PKB pathway is necessary for migration of porcine aortic endothelial cells, whereas the ERK pathway is not [186]. In contrast to insulin, IGF-I stimulates the synthesis of sulphated proteoglycans that are critical components of vascular basement membranes in both microvascular and macrovascular endothelial cells [163, 187].

As reviewed by Juul [174], IGF-I levels are decreased in liver disease and growth hormone deficiency. Similarly, IGF-I levels are low in Type 1 Diabetes and insulin-treated Type 2 Diabetes [188, 189]. In Type 1 Diabetes, the IGF system is abnormal even if glycemic control is normal or near normal [190]. This indicates that portal delivery of insulin is necessary to normalize the IGF levels. Low IGF-I levels are associated with increased risk of coronary artery disease [191] and chronic heart failure [192]. Decreased circulating levels of IGF-I account for poor prognosis in patients with manifest coronary artery disease [193]. Also, high IGF-I binding protein 3 levels in the circulation increase the risk of developing ischemic heart disease [191]. In obesity, free IGF-I levels are elevated. Increased levels of IGF-I have been reported to be associated with the pathogenesis of diabetic retinopathy [194].

IGF-I receptor

The IGF-I receptor is part of the same family of tyrosine kinase receptors to which the insulin receptor belongs [195]. The overall amino acid sequence homology exceeds 50%, reaching 84% in certain well-conserved areas. The IGF-I receptor is also heterotetramerical in structure; it exhibits the same βααβ-subunit structure as the insulin receptor (Figure 3). The activation of the α-subunit of the receptor also elicits trans-autophosphorylation of the opposite tyrosine kinase-containing β-subunit, followed by phosphorylation of the other β-subunit.

The IGF-I receptor binding domains supposedly are not as complex as those of the insulin receptor. The primary determinants of the IGF-I binding region are considered the cysteine-rich domain of the IGF-I holoreceptors and the C-terminal region [146]. Reduction of the disulfide bridges between the α-subunits results in two heterodimers with lower affinities for IGF-I than the one high-affinity binding site that the heterotetrameric IGF-I receptor displayed. The association of two heterodimers appears to result in one high-affinity binding site. The remaining IGF-I binding site has a lower affinity for IGF-I [196]. Affinity to bind insulin is approximately 100 to 1000 times lower than that for IGF-I. IGF-I receptor mRNA has been detected in human retinal endothelial cells [197]. In human glomerular endothelial cells, ligand-binding studies indicate the presence of IGF-I receptors [198].

Insulin/IGF-I hybrid receptors

The large degree of homology between insulin receptor and IGF-I receptor and the co-occurrence of the receptors in many cells are prerequisites for the formation of hybrid receptors. These hybrid receptors consist of one αβ heterodimer of the insulin receptor and one αβ heterodimer of the IGF-I receptor [199, 200] (Figure 3). Insulin/IGF-I hybrid receptors are present in a significant number of cell types and tissues. Thirty-six to 55% of total type 1 IGF-I receptors are reported to be hybrid receptors in a variety of human tissues, such as placenta, skeletal muscle, erythrocytes, leukocytes, and fibroblasts [201]. The proportion of total insulin receptors that were part of hybrids varied from 37 to 42% in placenta, skeletal muscle, erythrocytes, leukocytes, and fibroblasts with the exception of human adipose tissue, which only had 17% hybrid receptors. Another study has reported slightly higher proportions of hybrid receptors of total IGF-I receptors for placenta and skeletal muscle, 72 to 74% respectively [202]. The proportions of hybrid receptor of total IGF-I receptor in human heart, kidney, fat, and spleen reportedly vary from 53 to 87% [202]. The percentage of hybrid receptors expressed by a cell appears to be a function of the number of insulin receptors and IGF-I receptors [144]. It has been proposed that hybrid receptor formation is the result of random assembly of insulin receptor heterodimers and IGF-I receptor heterodimers [203]. A high proportion of hybrid receptors would therefore result from a large excess of one of the two receptors. Since insulin is known to regulate the expression of its own receptor [204], hybrid receptor formation could be affected by changes in the levels of insulin, *e.g.*, in insulin resistant states and obesity. In fact, this phenomenon has been described with an increase in the number of hybrid receptors in adipose tissue from patients with Type 2 Diabetes. This was coupled to impaired insulin sensitivity of the tissue *in vivo* and a decrease of insulin binding affinity *in vitro* [205].

Hybrid receptors behave more like IGF-I receptors than insulin receptors, *e.g.*, IGF-I has a greater ability to stimulate autophosphorylation than insulin. Also, hormone internalization and degradation by the hybrid receptor resembles more that of the IGF-1 receptor [144, 203, 206, 207]. Insulin cannot effectively displace bound IGF-I because of allosteric hindrance to insulin binding [143]. Hybrid receptors act differently when insulin receptor isoform A is involved than when isoform B is part of the hybrid. A hybrid receptor containing isoform A has been found to bind IGF-I, IGF-II, and insulin, whereas one containing isoform B predominantly binds IGF-I, binds IGF-II with low affinity and does not bind significantly to insulin [208]. Hybrid receptors made up of insulin receptor A and IGF-I receptor will therefore result in upregulation of IGF-I signaling since IGF-I signaling is also enhanced after stimulation with insulin. Hybrids consisting of IGF-I receptor and insulin receptor B will only respond to IGF-I. This activation pattern holds true for receptor phosphorylation but is also evident in cell proliferation and migration [208]. The presence of hybrid receptors on endothelial cells has not been studied.

Cellular signaling

The insulin receptor is thought to be a more effective transducer of metabolic signaling, whereas the IGF-I receptor preferably transduces mitogenic signals. However, both receptors seem to activate similar signaling molecules, such as insulin receptor substrate (IRS) proteins, PI-3K, MAPK and PKB [124]. The

MAPK-ERK pathway stimulates cell proliferation and migration and also involves Ras, Raf-1 and MEK [209, 210]. In insulin resistance and Type 2 Diabetes, stimulation of insulin receptor with insulin yields a blunted response of the PI-3K pathway but not of the MAPK pathway, which could contribute to atherosclerosis [211]. It is not yet clear how insulin and IGF-I activate the second messengers in such a way that they elicit differential effects. One study proposed that the different isoforms of the insulin receptor in pancreatic β-cells conveyed different functions in the cell and that was the result of different localization in the plasma membrane of the two isoforms [156].

A certain number of insulin receptors is required for differentiation of pre-adipocytes since overexpression of insulin receptors inhibits this process. In contrast, IGF-I receptor expression is not required for pre-adipocyte differentiation [154]. In this study of pre-adipocytes, IGF-I and insulin sometimes utilized only their cognate receptor *i.e.,* insulin uses insulin receptor to stimulate IRS-1 and PKB, whereas for activation of other signals, both receptors could be used. However, insulin receptor and IGF-I receptor-mediated signaling are not functionally redundant in pre-adipocytes. It appears that the ratio of IGF-I receptors to insulin receptors is more important for signaling than the total receptor number. Similarly, a limited ability of receptor compensation is apparent in murine knock-out models, where alternately the insulin receptor or the IGF-I receptor has been targeted [180]. Alternative or perhaps additional explanations for the specificity of the response are the differential phosphorylation of some substrates in response to insulin or IGF-I or the recruitment of specific substrates to the receptor, *i.e.,* IRS-1 to IGF-I receptor versus IRS-2 to the insulin receptor [212]. In human endothelial cells, the PI-3K/PKB pathway affects NO production after stimulation with insulin and IGF-I [165, 172].

Aim

The overall aim of this thesis is to study pathophysiological mechanisms in different aspects of Type 2 Diabetes. To fulfill this aim, the following sub-aims were pursued:

- To study voltage-gated calcium channels implicated in glucose-stimulated insulin secretion in INS-1 832/13 and INS-1 832/2 β-cells (Paper I)
- To study β-cell adaptation to insulin resistance induced by high-fat feeding of C57BL/6J mice (Paper II)
- To study glucose tolerance in RIP2-Cre mice, which are used for β-cell specific gene targeting (Paper III)
- To study the expression of the insulin receptor, IGF-I receptor and insulin/IGF-I hybrid receptor in human endothelial cells of different origin (Paper IV and V)

Models and Methods

Models

Cell models (Paper I, IV, V)

Rat INS-1 832/13 and 832/2 pancreatic β-cells were used for the study of the relative importance of different voltage-gated calcium channels (Paper I). These cells are derived from the INS-1 β-cell line [213]. INS-1 cells were originally isolated from a radiation-induced insulinoma, and respond with a 2- to 4-fold increase in insulin secretion after stimulation with glucose in the physiological range. Since INS-1 cells derive from a tumor, the initial clonal cell population becomes heterogeneous with time. This process negatively affects the robustness of glucose-stimulated insulin secretion. In order to identify subpopulations of cells with a stronger secretory response, INS-1 cells were stably transfected with a plasmid containing cDNA for human insulin and a neomycin resistance cassette [214]. The resultant discrete clones were subsequently analyzed for glucose-stimulated insulin secretion. The secretory response of 832/13 cells was 8- to 11-fold, whereas 832/2 cells hardly responded to glucose at all. Moreover, the amplifying pathway is present in 832/13 cells and these cells therefore serve as a model for a normal β-cell. On the other hand, 832/2 cells can be used as a model for defective β-cells.

Human umbilical vein endothelial cells (HUVEC) were used to study insulin receptors, IGF-I receptors and hybrid receptors in human endothelial cells (Paper IV), as were human coronary artery endothelial cells (HCAEC) (Paper V). These cells correspond to two different types of endothelial cells, one stemming from the venous circulation and the other one from the arterial circulation. HUVEC were obtained from umbilical cords as primary cells, and only kept in culture for 1 to 2 passages. HCAEC, which were obtained from Clonetics (San Diego, CA), were kept for up to 8 passages. Morphologically, both endothelium-derived cell types were identified as endothelial cells.

Animal models (Paper II, III)

As a model for insulin resistance, female C57BL/6J mice fed a high fat diet were used (Paper II). 60% of the calories in the diet were obtained from fat versus 10% in the control diet. The mice develop insulin resistance [215] and insufficient islet compensation [216] but do not develop frank Diabetes Mellitus. Instead they remain insulin resistant and glucose intolerant with compensatory hyperinsulinemia [217].

Rat insulin promoter 2-Cre recombinase (RIP2-Cre) mice were used for studies of glucose tolerance (Paper III). RIP2-Cre mice were originally created on a C57BL/6xDBA/2 genetic background; they are transgenic mice expressing the bacterial recombinase Cre driven by a 706 basepair fragment of the RIP2 [218]. The RIP-Cre mouse exhibits high expression levels of Cre in pancreatic β-cells [219], and low expression levels in the hypothalamus. The RIP2-Cre mice used in this study were bred onto a C57BL/6J genetic background by backcrossing for 14 generations, generating a near 100% pure C57BL/6J background.

Methods

RNA interference (Paper I)

RNA interference (RNAi) is a process whereby double-stranded RNA inhibits the expression of genes with a complementary nucleotide sequence through DNA/histone methylation, RNA degradation or translational blocking. It was first discovered in nematodes [220] but was later found to operate in most eukaryotic organisms [221, 222]. It was subsequently found that RNAi is executed by 21-23 nucleotide long double-stranded RNA molecules with a 3′-two nucleotide overhang on each strand [223]. The pathway probably serves as a form of innate immunity against viruses but may also play a role in regulation of development and genome maintenance. Scientists now use this naturally occurring process in the cell as an experimental tool in their studies.

The synthetic double-stranded short interfering RNA (siRNA) used in Paper I, were transfected into the cell, using a lipid-based transfection reagent. The siRNAs are supplied with a 3′-two nucleotide overhang on each strand, which resembles the structure of naturally occurring siRNA produced by Dicer, an RNase III enzyme, which constitutes the first step of RNAi. In the cell, the strands undergo strand separation. The siRNA strands bind to RNA-induced silencing complex (RISC), a multiprotein complex, where one strand, the antisense strand, guides the RISC complex to the target mRNA leading to mRNA degradation by nucleases (Figure 4).

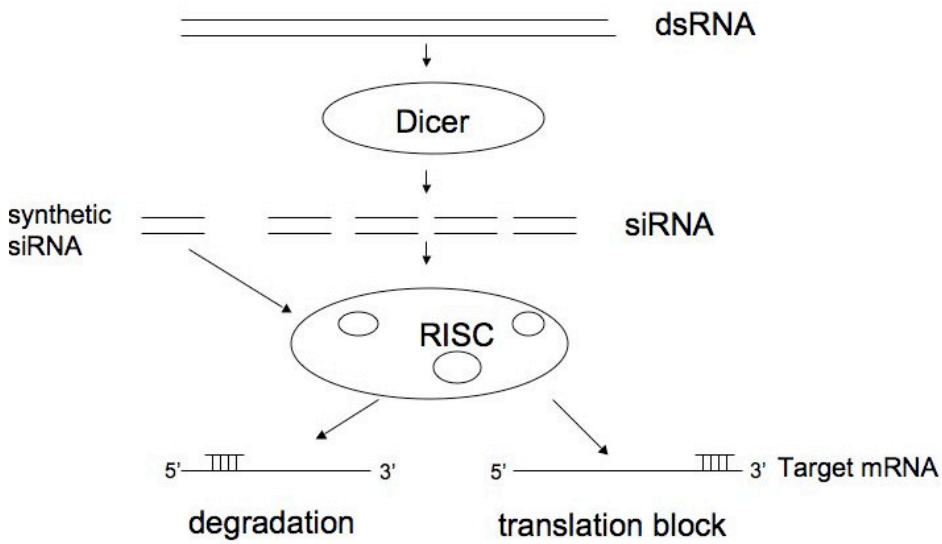

Figure 4. RNA interference. Double-stranded RNA (dsRNA) is cut into 21 nucleotide short interfering RNA (siRNA) by Dicer. This binds to the RNA-induced silencing complex (RISC) and the RISC complex, which is guided to the target messenger RNA (mRNA) resulting in its degradation. Figure is adapted from [224].

siRNA efficacy is influenced by two important factors. The first determinant is the transfection efficiency, which varies from cell to cell. INS-1 832/13 cells are normally difficult to transfect with plasmids, using standard

techniques such as electroporation or lipofection. However, since siRNA molecules are small, the transfection efficiency is remarkably higher than that of large DNA plasmids. Addition of a fluorescent marker to the siRNA and analysis of the cells post-transfection under a fluorescent microscope allows determination of transfection efficiency. Drawbacks to this are uncertainties with regard to the amount of fluorescent siRNA molecules needed to generate a signal, the exact localization of the fluorescent signal and whether an intracellular localization of the fluorescent signal reflects an activation of the RISC complex. The second determinant is the effectiveness of down-regulation achieved by the siRNA. This is determined by the location in the mRNA transcript of the sequence to which the siRNA will bind. However, clever algorithms for the design of siRNAs exist and their precision has improved continually, subsequently increasing the efficiency of gene silencing, e.g., mRNA degradation. In fact, downregulation by 95% of the mRNA level is possible to achieve. Analysis of the relative quantity of mRNA of the target sequence is therefore a crucial step in determining the efficacy of siRNA.

The concentration of siRNA added is an important factor. High concentrations of siRNA have been reported to cause off-target effects and saturation of the siRNA machinery [225]. These are defined as effects caused by unspecific binding of siRNAs. Indeed, as few as 11 matching nucleotides have been shown to be sufficient to silence non-target mRNAs [226]. Furthermore, binding to the 3'-UTR has been identified as one pathway to elicit off-target effects [227]. An optimal siRNA length of 21 nucleotides was determined to give rise to the least off-target effects [226], and this is the length of the siRNAs used in our study (Paper I).

Fluorescent measurements of intracellular Ca^{2+} concentrations (Paper I)

Fluorescence is a luminescence in which the molecular absorption of a photon in a fluorophore triggers the emission of another photon with a longer wavelength from the same fluorophore. Fluorescence can be used to study rapid changes in concentrations of ions. Fura-2 is a fluorophore, which can be used to measure changes in intracellular free Ca^{2+} ($[Ca^{2+}]_i$). Upon binding Ca^{2+}, fura-2 exhibits an absorption shift that can be observed by scanning the excitation spectrum between 300 and 400 nm, while monitoring the emission at ~510 nm. Indicators that show an excitation or emission spectral shift upon ion binding can be calibrated using a ratio of the fluorescence intensities measured at two different wavelengths. This results in the cancellation of artifactual variations in the fluorescence signal, such as photobleaching, leakage of the indicator, non-uniform indicator distribution, that might otherwise be misinterpreted as changes in ion concentration.

In Paper I, Fura-2 acetoxymethyl (AM) was used. Fura-2 AM is a cell-permeant uncharged Ca^{2+} indicator that can be loaded passively into cells. Intracellularly, the AM group is cleaved off and this results in a charged form of Fura-2, which is cell-impermeant. The cells were held in a perfusion chamber and a mercury lamp was used to excite light alternately at 350 and 380 nm, the emitted light was collected at 510 nm in a photomultiplier detector. The $[Ca^{2+}]_i$ was calculated using equation 5 of Grynkiewicz *et al.* [228] with a K_d of 224 nM.

Patch clamp (Paper I)

The electrophysiological properties of a single cell can be studied with the patch-clamp technique, which was invented in 1978 by Neher *et al.* [228, 229]. This method can be used to study (i) the recording of currents through the cell membrane, (ii) measurements of membrane potential and (iii) recordings of exocytotic responses that are monitored as increases in cell capacitance. Patch clamp traditionally uses a heat-polished glass pipette with a smooth surfaced open tip, which is filled with pipette solution. This pipette is pressed to the cell membrane forming a tight seal (giga-ohm seal), coupling the cell to an electrical circuit. In the whole-cell configuration, the cell membrane is ruptured by suction at the point where the pipette is patched to the cell. The suction causes an opening in the cell membrane and the cytosolic contents are replaced by the pipette solution. When currents are measured (i), the voltage is clamped and stimulating pulses are applied from a negative resting potential. The opening or closure of an ion channel will lead to variations in the holding current, which can be quantified. However, when the currents are clamped, the voltage (membrane potential, (ii)) can be measured. In measuring exocytotic events (iii), the voltage is clamped by application of a sinusoidal voltage and the changes in the cell surface area are monitored as increases in cell capacitance. Fusion of a secretory granule will temporarily increase the cell surface area. Both current and cell capacitance measurements were used in Paper I.

Metabolic assays (Paper I, II, and III)

The studies performed with the focus on pancreatic β-cells all use fuel-stimulated insulin secretion as an end-point for determining the importance of the mechanisms studied. Insulin secretion assays were conducted with either INS-1 832/13 and 832/2 cells (Paper I) and mouse islets (Paper II, III). Stimulation with glucose or other metabolic fuels was performed for one hour and the insulin accumulated in the medium was measured by radioimmunoassay.

In Paper II, an intravenous glucose tolerance test (IVGTT) was carried out. In an IVGTT, glucose is administered intravenously and subsequent blood samples are taken to determine plasma glucose and insulin levels. This will report the rate of glucose clearance from the circulation. The determination of insulin concentrations allows discrimination of the cause of glucose intolerance: insulin resistance or impaired insulin secretion.

Fuel oxidation experiments were carried out (Paper II). Mouse islets were incubated with different radioactively labeled fuels and the production of radioactively labeled CO_2 was determined. These experiments are used to determine which fuels the islets utilize and whether high-fat diet changes the fuel use.

Receptor identification (Paper IV and V)

In the study of receptors, both the presence of mRNA and protein were determined. Determining the presence of mRNA and protein cannot identify the insulin/IGF-I hybrid receptor, because the receptor consists of one half of each receptor. However, by immunoprecipitating the cell lysate with an antibody specific for insulin receptor, one isolates both the insulin receptor and the insulin/IGF-I hybrid receptor. The subunits subsequently get separated in the denaturating conditions of the SDS-PAGE and the individual subunits can be identified on immunoblot. The same process can be used to isolate IGF-I receptor and hybrid receptor. The use of different (monoclonal) antibodies in the

immunoprecipitation and the immunoblotting increases the specificity of the process.

Another way to discern the presence of receptors is by performing substrate-binding studies. These are performed by incubation of cells with radioactively labeled substrate at a constant concentration and unlabeled substrate at different concentrations. Homologous insulin receptors will show a lower IC_{50} value, *i.e.*, the concentration needed to give half-maximal displacement of the radioactively labeled substrate, for insulin than for IGF-I. The same holds true for homologous IGF-I receptors. Hybrid receptors cannot be identified this way although comparing IC_{50} values for both substrates with each radioactively labeled substrate can be used to derive an indication for their presence. If the IC_{50} values are closer together, part of the binding might be explained by binding to hybrid receptors whereas if the IC_{50} values are far apart, homologous receptors are present to a greater degree.

The identification of the receptor protein in cell lysate is not a measure of the activity of the receptor. Short-duration receptor stimulation by different concentrations of insulin or IGF-I and subsequent immunoprecipitation of the receptor followed by immunoblot with antibody for tyrosine phosphorylation, is a way to show the functionality of the receptor.

Results

The role of $Ca_V1.2$ in insulin secretion from INS-I 832/13 cells (Paper I)

An aim of this study was to determine which calcium channel is critical for glucose-stimulated insulin secretion in 832/13 cells and whether the 832/13 clone differed from the 832/2 clone in the expression of voltage-gated calcium channels. A potential difference in voltage-gated calcium channels could be one of the explanations for the disparity in the extent of glucose-stimulated insulin secretion between these two cell clones [214].

mRNA expression levels for $Ca_V1.2$, $Ca_V1.3$ and $Ca_V2.3$ did not differ between 832/13 and 832/2 cells. However, the expression levels for $Ca_V1.2$ were two orders of magnitude higher than those of $Ca_V1.3$ and $Ca_V2.3$, which were similar. Glucose-stimulated insulin secretion was very different in 832/13 cells versus 832/2 cells: the fold response of 832/13 cells was 16-fold, whereas it was virtually absent in 832/2 cells. Stimulation of both clones with high 35 mM KCl, bypassing the metabolic closure of K_{ATP}-channels, resulted in stimulation of insulin secretion although the extent of the increase was more pronounced in 832/13 cells. Dihydropyridines such as isradipine can be used to block calcium currents through $Ca_V1.2$ and $Ca_V1.3$. Co-incubation of isradipine and 16.7 mM glucose decreased insulin secretion to basal levels in 832/13 cells, whereas it had no significant effect on insulin secretion from 832/2 cells. Insulin secretion stimulated by high K^+ and diazoxide could also be inhibited by isradipine in both 832/13 and 832/2 cells. Currents through $Ca_V2.3$ can be inhibited by the peptide SNX-482. There was no significant effect of SNX-482 on insulin secretion in either cell clone studied.

$[Ca^{2+}]_i$ was studied in these cells under the same conditions with fluorescent measurements of the calcium fluorophore Fura-2 AM. Elevated glucose (15 mM) increased in calcium influx into 832/13 cells and the frequency of calcium spikes in these cells. 832/2 cells did not show any increase in calcium spikes and $[Ca^{2+}]_i$ was not significantly raised. Isradipine brought the frequency of calcium spikes in 832/13 cells back to the basal level and inhibited an increase in $[Ca^{2+}]_i$ whereas it did not affect $[Ca^{2+}]_i$ in 832/2 cells. However, the $[Ca^{2+}]_i$ increased to the same extent in 832/13 and 832/2 cells in response to 35 mM KCl, indicating the presence of functional voltage-gated calcium channels in 832/2 cells. SNX-482 did not affect $[Ca^{2+}]_i$ in either cell clone.

Since isradipine inhibits both $Ca_V1.2$ and $Ca_V1.3$, and the expression levels of $Ca_V1.2$ and $Ca_V1.3$ were so discordant, we used siRNA against $Ca_V1.2$ to determine the significance of this calcium channel for insulin secretion in 832/13 cells. The transfection efficiency of the siRNA was determined by transfection of negative control siRNA coupled to the fluorescent marker Alexa-488 and was approximately 75%. $Ca_V1.2$ mRNA expression levels were decreased by 65% while $Ca_V1.3$ and $Ca_V2.3$ mRNA levels were not affected by transfection of $Ca_V1.2$ siRNA. Glucose-stimulated insulin secretion in siRNA-treated cells was decreased by approximately 50% and could be further reduced by addition of isradipine. Calcium currents in siRNA-treated cells were studied with patch clamp in the standard whole-cell configuration. The maximal peak-current was reduced by 60% in siRNA treated-cells whereas the Na^+-current was not affected. The effect of $Ca_V1.2$ siRNA on exocytosis was also studied. Exocytosis was evoked by a train of ten 500-ms depolarizations from -

70 mV to 0 mV and was found to be decreased by 50% in $Ca_V1.2$ siRNA-treated cells.

β-cell adaptation to insulin resistance in a mouse high-fat diet model (Paper II)

C57BL/6J mice, a model for insulin resistance when kept on a high-fat diet, were fed a high-fat (HF) diet consisting of 60% fat, 20% protein and 20% carbohydrates or control diet with 20% protein, 70% carbohydrates and 10% fat for 12 weeks. After 5 weeks on the HF diet, plasma insulin levels were already dramatically increased and continued to rise. However, plasma glucose levels were not significantly elevated at any point indicating compensated insulin resistance in HF mice. Ten weeks into the HF diet, an IVGTT showed retarded glucose clearance in HF mice, with basal hyperinsulinemia and greater amounts of insulin secreted during the IVGTT, again indicating compensated insulin resistance and glucose intolerance in this mouse model.

Islets from HF mice had increased basal insulin secretion and only a slight increase in insulin secretion in response to 15 mM glucose. When stimulated with high K^+ and diazoxide (K_{ATP}-independent condition), HF islets secreted more insulin than control islets. However, HF islets did not increase their secretory response further after addition of glucose to high K^+ and diazoxide in contrast to control islets, indicating a loss of the amplifying pathway. Insulin secretion was also enhanced after stimulation with palmitate and high glucose in HF islets versus control islets although there was no difference at basal glucose concentrations. In contrast, under K_{ATP}-independent conditions supplemented with palmitate, HF islets exhibited a large increase in insulin secretion compared to control islets. This disparity remained after addition of high glucose although the difference in insulin secretion between HF and control islets was less than at basal glucose concentrations. Other secretagogues, such as BCH (a non-metabolizable form of leucine which stimulates glutamate dehydrogenase) stimulated insulin secretion in HF islets significantly more at basal glucose concentrations than in control islets. BCH in combination with glutamine also stimulated insulin secretion more strongly in HF islets than in controls. This also held true for methylsuccinate, which may cause insulin secretion via an anaplerotic pathway, and α-ketoisocaproic acid, which can be oxidized in the mitochondria and form acetyl-CoA but also be transaminated to leucine, an allosteric activator of glutamate dehydrogenase. Glutamine alone caused vigorous insulin secretion from HF islets under K_{ATP} dependent conditions. Dimethyl-glutamate elicited an increase in insulin secretion in HF islets at high glucose concentrations that was twice that of control islets. In short, HF islets have an exaggerated secretory response to mitochondrial fuels. Fuel oxidation was also affected in HF mice: glucose oxidation in response to high glucose was decreased, whereas oxidation of palmitate and glutamine were increased at basal glucose concentrations.

The decrease in glucose oxidation but not in mitochondrial fuel oxidation led us to examine Glut2 localization and mRNA expression levels. The mRNA levels for Glut2 were not different in HF and control islets but Glut2 immunofluorescence, however, in HF β-cells was diffuse or decreased compared to control β-cells, which displayed Glut2 immunofluorescence at the plasma membrane. Lipid droplets were also present in the β-cells of HF mice but were not observed in control mice. Ultrastructural studies showed an increase in mass, but not in number, of mitochondria, in HF mice *versus* control

mice. We attempted to elucidate the factors involved in mitochondrial biogenesis in our model; the transcription factors peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α), PGC-1β, nuclear respiratory factor-1 (NRF1), mitochondrial transcription factor A (TFAM), and the nuclear-encoded mitochondrial protein cytochrome C and the mitochondria-encoded mitochondrial protein cytochrome c oxidase IV (COXIV) mRNA levels were determined. PGC-1 is thought to act in concert with other transcription factors and thereby stimulate the transcription of a number of genes such as *tfam*. However, we could not determine significant differences in mRNA expression levels of any of these factors after 12 weeks of HF feeding. Previously, high-fat diet has been demonstrated to decrease genes for oxidative phosporylation proteins in skeletal muscle in the same mouse model, although mtDNA copy number was not altered [230]. A significant decrease in PGC-1α and PGC-1β but not NRF1 or 2 mRNA expression was reported after 3 weeks of HF feeding. Since we determined mRNA expression levels after 12 weeks, we hypothesized that the increase in mRNA levels occurred at an earlier time-point of HF feeding. However, expression levels for the genes studied did not differ between HF islets and control islets at 2, 6, or 8 weeks. The increase in β-cell mitochondrial mass could be due to increased mRNA levels of other transcription factors as well as changes in mRNA translation into protein or increased protein stability.

Glucose tolerance in RIP2 C57BL/6J mice (Paper III)

In this study, we compared glucose tolerance in female RIP2-Cre heterozygote mice, which originally came from the laboratory of Professor Mark A Magnuson. Since 2002, the mice were backcrossed in our laboratory for 14 generations onto a C57BL/6J background, yielding a 99.9% C57BL/6J background. C57BL/6J mice were obtained from the Taconic facility, which attained these mice in 1991 from the NIH Animal Genetic Resource and has since inbred these mice. C57BL/6J littermate mice served as wild-type (WT) control mice.

At 12 weeks, fasted plasma glucose and insulin levels were similar in RIP2-Cre and WT mice. Glucose clearance and insulin levels during the IVGTT did not differ either. *In vitro* static incubations with islets isolated from 28 week-old mice with glucose at different concentrations; K_{ATP}-independent conditions with glucose; palmitate; GLP-1; carbacholine; and α-ketoisocaproic acid, all provoked a similar secretory response in RIP2-Cre and WT mice. β-cell mass in pancreatic sections of 28-week-old mice was not different in the two genotypes.

Glucose intolerance due to a five-exon deletion, exon 7 to 11, in the *nnt* gene was recently reported in C57BL/6J mice [59]. Nnt protein is completely lost in the mice displaying this deletion. The glucose intolerance may arise as a result of impaired insulin secretion due to the absence of Nnt, and develops spontaneously. We therefore examined the *nnt* gene in our RIP2-Cre and WT mice on the C57BL/6J background. However, both exon 8 and exon 11 were found to be present, indicating that the five-exon deletion was not present in C57BL/6J mice from the Taconic facility.

Insulin receptors and IGF-I receptors in human endothelial cells (Paper IV and V)

Papers IV and V focus on insulin receptor, IGF-I receptor, and insulin/IGF-I hybrid receptor in human endothelial cells. Both endothelial cell types studied show similar patterns in the expression and ligand binding of the receptors.

IGF-I receptor mRNA levels were higher than those of insulin receptor in human endothelial cells, with HCAEC showing a more pronounced difference in the mRNA expression levels than HUVEC. HUVEC IGF-I mRNA levels exhibited a tendency to decrease during culture although this difference was not statistically significant.

In binding studies, the affinity of the receptors for the ligands was determined and through ligand binding an indication for protein levels of the respective receptors could be obtained. Insulin and IGF-I were the ligands used, with the addition of the insulin analogue glargine for HCAEC. The specific binding of radiolabeled IGF-I was higher than for radiolabeled insulin indicating that the number of IGF-I receptors was higher than the number of insulin receptors, confirming the data obtained for receptor mRNA levels. Furthermore, comparison of the IC_{50} values for displacement of ^{125}I-IGF-I by IGF-I and insulin revealed that IGF-I was several orders of magnitude more efficient in displacing radiolabeled IGF-I than insulin. Again the difference was more pronounced in HCAEC than in HUVEC. The IC_{50} value for glargine, the insulin analogue, was lower than that for insulin but higher than that of IGF-I. Comparatively, the IC_{50} values for displacement of ^{125}I-insulin by insulin and IGF-I did not differ as much, with insulin only 30-fold more efficient at displacing radiolabeled insulin than IGF-I in HUVEC. In HCAEC, the specific binding of ^{125}I-insulin was too low to enable calculation of IC_{50} values.

The β-subunits of insulin receptor and IGF-I receptor are very similar in size, differing only by 2 kDa; insulin receptor β-subunit is 95 kDa and IGF-I receptor β-subunit is 97 kDa in size. Careful separation of immunoprecipitated proteins on Western blot, will lead to the presence of two distinct bands located closely together when hybrid receptors are present. In both endothelial cell types, distinct bands for insulin receptor β-subunit and IGF-I receptor β-subunit could be identified with a variety of monoclonal and polyclonal antibodies. Evidence for hybrid receptors was also found after immunoprecipitation of the insulin receptor and Western blot with an antibody for the IGF-I receptor and *vice versa*.

Activation of the receptor tyrosine kinase was studied thereafter by 10-minute stimulations of receptor phosphorylation by different concentrations of ligand in the presence of a tyrosine phosphatase inhibitor. The cell lysate was immunoprecipitated with an antibody against insulin receptor or IGF-I receptor and immunoblotted with an antibody specific for phosphorylated tyrosine kinase. IGF-I elicited IGF-I receptor phosphorylations at concentrations from 0.1 to 10 nM. Stimulation of the insulin receptor with 1 to 10 nM insulin resulted in insulin receptor phosporylation. However, insulin receptor phosphorylation also resulted from stimulation with IGF-I at 1 to 10 nM. The IGF-I receptor could be phosphorylated by insulin at a concentration ≥ 1 μM, a concentration previously shown to be effective in stimulating IGF-I receptor phosphorylation [231].

Discussion

Insulin secretion in INS-1 832/13 cells is to a large extent dependent on dihydropyridine-sensitive voltage-gated calcium channels. Our study indicates that $Ca_V1.2$ is the main Ca^{2+}-channel accounting for glucose-stimulated insulin secretion in these cells. This notion is supported by the mRNA expression levels of $Ca_V1.2$ in 832/13 cells, which were two orders of magnitude higher than of $Ca_V1.3$ and $Ca_V2.3$. Furthermore, RNAi of $Ca_V1.2$ decreased mRNA expression levels by 65%; concurrently, calcium currents, exocytotic events and glucose-stimulated insulin secretion were reduced by 50%. The remaining $Ca_V1.2$ expression levels can be explained by the transfection efficiency attained in our experiments, which was 75%, suggesting that 25% of the cells were untransfected and thereby expressed 100% $Ca_V1.2$ mRNA. Moreover, the knockdown efficiency of the siRNAs used is not 100% and therefore, this also contributes to remaining $Ca_V1.2$ mRNA expression. The effect on protein levels was not studied but is likely not to exceed the levels of down-regulation of mRNA levels. $Ca_V1.2$ is also the calcium channel underlying glucose-stimulated insulin secretion in mouse β-cells [88]. In humans, a rare mutation in $Ca_V1.2$ is associated with a wide variety of symptoms, among which is intermittent hypoglycemia [123], implicating $Ca_V1.2$ in human insulin secretion as well. In mouse, $Ca_V2.3$ has been implicated in second-phase insulin secretion [99] but this has not consistently been repeated in rat β-cells [100, 101, 232].

Furthermore, the lack of glucose-stimulated insulin secretion in 832/2 cells does not appear to be due to difference in expression levels, or functionality of voltage-gated calcium channels. Both cell clones expressed similar mRNA expression levels, and K^+-stimulated insulin secretion in 832/2 cells could be inhibited by isradipine, indicating the presence of functional dihydropyridine-sensitive calcium channels. Therefore, the lack of glucose-stimulated insulin secretion in 832/2 is more likely to be due to changes in processes preceding the opening of voltage-gated calcium channels. Indeed, earlier studies have suggested that pyruvate cycling [233] and signaling in the cAMP/protein kinase A pathway [234] are less pronounced in 832/2 cells versus 832/13 cells.

Insulin secretion was increased in our model of insulin resistance: C57BL/6J mice on a HF diet. In fact, disturbances in glucose tolerance and insulin levels can be identified as early as one week after start of the diet [216]. However, the mice do not develop overt Diabetes Mellitus since the β-cells secrete sufficient amounts of insulin to compensate for the insulin resistance. In our study, the β-cells of the HF-fed mice adapted with a shift from glucose oxidation to that of free fatty acids and amino acids. This is in accordance with results of studies in two other animal models of insulin resistance: hypersecretion of insulin in response to α-ketoisocaproic acid in the Brattleboro mouse [235] and increased lipolysis and fatty acid signaling in the Zucker fatty rat [236]. The high amount of lipids in the β-cells of the HF mice might interfere with the glucose transporter Glut2, as has been reported earlier [138], to which impaired oxidation of glucose may be attributed. The adaptation also expresses itself as an increase in mitochondrial mass in the β-cells. Although we could not identify the transcription factors responsible for the increase in mitochondrial mass, changes in mitochondrial mass in β-cells have previously been reported in β-cells from human Type 2 Diabetes patients [237].

Recently, reports on retarded glucose elimination due to impaired insulin secretion in C57BL/6J on a normal diet and RIP2-Cre mice have been published [59, 218]. Spontaneous development of glucose intolerance could affect the conclusions from the HF diet study (Paper II) as well as the results of other studies that use these mouse models. A five-exon deletion in the *nnt* gene was reported to cause glucose intolerance in C57BL/6J mice [59]. The Nnt protein is localized to the inner mitochondrial membrane and is a major generator of NADPH. It couples NADPH production to the production of reactive oxygen species generated by the electron transport chain. The deletion in the gene gives rise to the loss of Nnt protein, which results in the stimulation of the activity of UCP-2 by reactive oxygen species. This leads to increased proton leakage across the inner mitochondrial membrane, which reduces the electronmotive force and thereby ATP synthesis [62]. However, C57BL/6J mice obtained from the Taconic facility used to breed the RIP2-Cre mouse onto, did not display the five-exon deletion. This could explain the lack of glucose intolerance due to impaired insulin secretion in the WT mice used in Paper III. It also validates the results obtained in the HF diet study (Paper II). This indicates once again the difficulty in comparing results from animal models in different laboratories since small differences, with potentially large consequences, might be present even between animals from the "same" mouse strain in different facilities. The RIP2-Cre mouse has been bred on different genetic backgrounds in different laboratories. The RIP2-Cre mouse used in paper III is on a 99.9% C57BL/6J background. The absence of any significant differences in *in vivo* and *in vitro* insulin secretion, and β-cell mass between the RIP2-Cre and WT littermates studied, argues against a major toxic effect of high concentrations of the Cre recombinase itself in β-cells. β-cell mass has also been examined in RIP2-Cre mice in the laboratory of our collaborator Dr Michael Ristow. Interestingly, they find dynamic increases and decreases of β-cell mass over time [238]; at this point, it is not known whether their line of C57BL/6J mice harbors the five-exon deletion in the *nnt* gene.

Insulin exerts its actions via binding to the insulin receptor. If present in very high concentrations, insulin is able to bind to the IGF-I receptor, as shown by *in vitro* studies [231]. We have studied the presence of insulin receptors, IGF-I receptors and insulin/IGF-I hybrid receptors in human endothelial cells. The endothelium is affected in complications of Diabetes Mellitus both in small and large vessels. An inequality in the number of insulin receptors versus IGF-I receptors is thought to contribute to the expression of hybrid receptors, which have a higher affinity for IGF-I than for insulin and thereby activate signaling pathways associated with the IGF-I receptor, decreasing the effect of insulin. We have several indications of the presence of hybrid receptors in endothelial cells of different origins (Paper IV and V) and that these receptors are activated by IGF-I but not insulin. Hybrid receptors and the higher abundance of IGF-I receptors versus insulin receptors reflect the importance of IGF-I in human endothelial cells. Since IGF-I levels in insulin-treated Type 2 Diabetes are low [174], decreased signaling through the IGF-I receptor and through the hybrid receptor may affect NO production in endothelial cells [165, 172] and contribute to cardiovascular disease. Although IGF-I levels are normal in Type 2 Diabetes patients with good glycemic control, they tend to decrease in patients with poor glycemic control [239]. In cardiovascular disease, IGF-I levels are decreased and it has been suggested that in Type 2 Diabetes patients, de-

creased IGF-I levels may be a causal factor in the pathogenesis of cardiovascular disease [240].

Summary and Conclusions

In this thesis, we have studied pathophysiological mechanisms in Type 2 Diabetes Mellitus. The first part of the thesis focused on insulin secretion both in the normal and the insulin resistant state. The focus in the second part was on insulin and IGF-I receptors in human endothelial cells, which are involved in complications of diabetes.

In the rat pancreatic β-cell line INS-1 832/13, the dihydropyridine-sensitive voltage-gated calcium channel $Ca_V1.2$ is essential for glucose-stimulated insulin secretion. A minor role for $Ca_V1.3$ cannot be excluded but $Ca_V2.3$ does not appear to affect insulin secretion in these cells. INS-1 832/2 cells, which have a poor secretory response to glucose, expressed the same calcium channels at similar levels. Therefore, processes preceding the opening of the voltage-gated calcium channels are likely to be responsible for the poor secretory response.

β-cells in the insulin resistance model of the high-fat diet-fed C57BL/6J mouse adapt by increasing mitochondrial mass and by switching to the use of fuels other than glucose. This adaptation is associated with the increased secretion of insulin leading to hyperinsulinemia and preventing the development of overt diabetes in this mouse model.

Glucose tolerance and insulin secretion in the RIP2-Cre mice are not different from wild type C57BL/6J mice. The strain of C57BL/6J used in our facility was demonstrated to express two of the exons implicated in the five-exon deletion of the *nnt* gene. The mice are thereby not affected by the decrease in insulin secretion associated with this mutation. Furthermore, expression of the Cre-recombinase in β-cells in this mouse strain does not affect glucose tolerance.

Human endothelial cells express receptors for insulin, IGF-I and insulin/IGF-I hybrid receptors. IGF-I receptors are expressed to a higher extent, giving rise to the assembly of hybrid receptors. IGF-I activates receptor phosphorylation at lower concentrations than insulin activates its own receptors in these cells. IGF-I appears to be able to activate hybrid receptor phosphorylation as well.

Populärvetenskaplig sammanfattning

Förekomsten av Diabetes Mellitus (sockersjuka), en grupp sjukdomar som kännetecknas av förhöjda halter av glukos (socker) i blodet, ökar över hela världen. Till år 2025 har antalet människor i världen som lider av diabetes ökat till 300 miljoner. Den största ökningen i antal kommer att äga rum i Asien, framför allt i Indien och Kina. Ungefär 90 % av alla patienter med diabetes har fått diagnosen Typ 2 Diabetes. Typ 2 Diabetes är förknippad med fetma och brist på fysisk aktivitet. De kvarstående 10 % lider av andra former av diabetes, framför allt Typ 1 Diabetes.

I friska individer hålls blodglukoshalten inom väldefinierade gränser. Insulin och glukagon är de hormoner som huvudsakligen upprätthåller denna reglering av blodglukoshalter. Insulinhalterna i blodet ökar när blodglukoshalterna stiger efter födointag. Insulin tillverkas i β-celler som finns i endokrina (hormonfrisättande) öar i bukspottkörteln. Insulinfrisättning är en invecklad process. β-cellerna, som fungerar som ett slags glukossensorer, tar upp glukos från blodet och spjälkar det i en process som ger upphov till energi. Den energi bildas som ATP, vilket är generell energibärare i cellen, i cellernas mitokondrier. Mitokondrier är de enheter i cellerna vars huvuduppgift är att producera energi. ATP transporteras ut från mitokondrierna till cytosolen, en vätska som omgiver de olika delar i cellerna, och detta stänger en ATP-känslig kaliumjonkanal som finns i cellmembranet. Detta medför en förändring i den elektriska spänningen över cellmembranet, som i sin tur öppnar spänningskänsliga kalciumjonkanaler. Kalcium flödar in i cellen och det signalerar till β-cellerna att insulin ska frisättas till blodet. Insulin kan binda till insulinreceptorer, ungefär som en nyckel passar i ett lås. Receptorerna sitter på cellmembranet av celler i målvävnader och ger upphov till en signal inuti målcellen. Insulins målvävnader är skelettmuskler, fettväv, och lever. Insulins bindning till receptorer på muskler och fett stimulerar glukosupptag från blodet, och i lever minskas glukosproduktionen. Glukagons huvuduppgift är att motverka insulin. Glukagon frisätts från α-celler, som är systerceller till β-cellerna i de endokrina öarna i bukspottkörteln. När blodglukoshalterna sjunker, signalerar glukagon till levern att öka glukosproduktionen. Frisättning av glukos från intracellulära depåer i muskler stimuleras också av glukagon. Således försäkras tillgång på glukos för kroppens celler mellan måltider.

Typ 2 Diabetes uppstår på grund av två defekter i regleringen av blodglukoshalterna. Den ena är otillräcklig insulinfrisättning från β-cellerna och den andra är en minskad effekt av insulin i målvävnaderna, en process som kallas för insulinresistens. Resultatet av dessa defekter visas i förhöjda blodglukoshalter som i första hand behandlas med diet och ökad motion, därefter med läkemedel som stimulerar insulinfrisättning eller ökar insulinets effektivitet i målvävnaderna, och till slut med insulin. Blodglukoshalterna i Typ 2 Diabetes patienterna ligger ändå ofta över den genomsnittliga i den friska populationen. Denna ökning är en av de orsakerna till utveckling av komplikationerna i diabetes. Dessa består av kardiovaskulära sjukdomar, njurproblem och synfel. Komplikationerna utvecklas långsamt i de flesta patienter, men kardiovaskulära komplikationer är en av de största dödsorsakerna i Typ 2 Diabetes.

I denna avhandling har vi försökt att identifiera mekanismer som bidrar till utvecklingen av Typ 2 Diabetes och till utvecklingen av komplikationer till Typ 2 Diabetes.

Det första arbetet fokuserar på de spänningskänsliga kalciumkanalerna, som är viktiga för insulinfrisättning i β-celler från råtta. Det finns olika typer av spänningskänsliga kalciumkanaler och deras betydelse för insulinfrisättning skiljer sig mellan olika djurarter och mellan olika modeller i samma art. I våra β-celler var kalciumkanalen $Ca_V1.2$ mest väsentlig för glukosstimulerad insulinfrisättning. Vi jämförde dessa β-celler med sådana som inte visar en glukosstimulerad insulinfrisättning och som kan användas som en modell för β-celler i Typ 2 Diabetes. Vi kunde inte hitta skillnader i uttryck av spänningskänsliga kalciumkanaler mellan dessa två celler. Vi misstänker att de skillnaderna i insulinfrisättning beror på processer som föregriper öppningen av de spänningskänsliga kalciumkanaler.

I nästa arbete undersökte vi mekanismer bakom anpassningen av β-cellerna vid insulinresistens. Vi använde oss av möss som givits fetkost, där en hög andel av kalorierna kommer från fett, som gjorde mössen insulinresistenta. Vid insulinresistens behövs högre halter av insulin för att upprätthålla en normal blodglukoshalt. Detta medför att β-cellerna måste frisätta mer insulin och när det inte sker uppstår Typ 2 Diabetes. De möss som användes här ökar insulinfrisättning markant och utvecklar alltså ingen diabetes. Vi ville undersöka på vilket sätt β-cellerna i dessa möss genomförde denna omställning. Resultaten visar att β-cellerna började frisätta insulin i respons till andra ”bränslen” än glukos som fett och aminosyror (protein). Detta kan vara en följd av de höga fetthalterna i cellerna. Fettet ansamlades i β-cellerna vilket kunde ses som fettdroppar när cellerna studerades närmare med elektronmikroskop. Denna höga fetthalt i cellerna kan påverka glukostransportproteiner så att glukostransport in i celler minskar. β-cellerna i dessa möss som erhållit fetkost hade också en större mitokondrieyta, vilket kan vara kopplad till den ökade insulinfrisättningen.

Den musstam som vi använde i vår fetkostmodell kan spontant utveckla glukos intolerans enligt ny forskning. Glukosintolerans är ett tillstånd där insulinfrisättningen är otillräcklig när glukos tillförs utan att patienten har utvecklad diabetes. Denna glukosintolerans skulle kunna uppstå till följd av en mutation i en gen, vars funktion är inblandad i glukosspjälkning och ATP produktion som behövs för glukosstimulerad insulinfrisättning. Vi undersökte förekomsten av mutationen i de möss som användes i fetkoststudien. Mutationen fanns inte i denna musstam och glukostoleransen var normal i dessa möss. Vi studerade även en relaterad musstam, som ofta används för att studera effekterna av knockout av vissa gener specifikt i β-celler. Om denna mus utvecklar glukosintolerans spontant skulle den inte vara lämplig för att studera effekten av en gen på glukostolerans eller insulinfrisättning. Denna stam hade dock inte den mutationen och var inte heller glukosintolerant.

Slutligen har vi studerat insulins effekter. Insulin utövar sin effekt på målvävnader via inbindning till sin receptor. Insulin receptorn är en medlem av en familj av liknande receptorer. Receptorn för insulin-like growth factor-I (IGF-I) tillhör samma familj. IGF-I receptorn aktiveras oftast av IGF-I, en hormon som liknar insulin. IGF-I har en viktig funktion i tillväxt och utveckling men det spelar även en roll i reglering av blodglukoshalterna. Frisättning av insulin från β-celler i blodet framkallar IGF-I frisättning från levern. IGF-I halterna är påverkade i Diabetes Mellitus. Insulinreceptorn och IGF-I receptorn initierar både unika och överlappande effekter. Båda receptorer är till sin struktur, vilket kan ge upphov till bildning av insulin/IGF-I hybridreceptorer. Hybridreceptorn är framställd ur en halv insulin receptor och en halv IGF-I receptor. Signalen från en hybrid receptor liknar signalen från en IGF-I recep-

tor även om receptorn aktiveras av insulin. Eftersom komplikationer i Diabetes Mellitus ofta är sekundärt till problem i blodkärl ville vi kartlägga om receptorer för insulin och IGF-I finns i humana endotelceller, de celler som utgör det inre lagret av blodkärlsväggen. Antalet IGF-I receptorer var högre än insulin receptorer i dessa celler. Insulin kunde aktivera insulin receptorn och IGF-I kunde stimulera sin receptor. Vi hittade belägg för förekomsten av insulin/IGF-I hybrid receptorer och de verkar bli aktiverade av IGF-I. Dessa fynd antyder att IGF-I spelar en roll i utvecklingen av vaskulära komplikationer i Diabetes Mellitus.

Vi har således studerat olika aspekter av Typ 2 Diabetes, vilka bidrar dock till det stora pusslet som utgör sjukdomen Typ 2 Diabetes.

Popular Scientific Summary

The incidence of Diabetes Mellitus, a family of diseases characterized by high levels of blood glucose, is globally on the rise. The number of people suffering from this disease will increase to 300 million by 2025 with the greatest increase to occur in Asia, mainly India and China. The largest proportion of people with Diabetes Mellitus, around 90%, suffers from Type 2 Diabetes, which is associated with obesity and lack of physical activity. The remainder suffers from the other forms of Diabetes Mellitus with Type 1 Diabetes as the leading cause in this group.

Blood glucose levels are being held within strict limits in healthy individuals. The hormones mainly responsible for this regulation are insulin and glucagon. Insulin levels in the blood rise in response to increases in blood glucose levels after a meal. The cells responsible for the production of insulin are the β-cells, located in islets of endocrine (hormone-releasing) cells in the pancreas. Insulin release is a highly complex process. The β-cells, which function as blood glucose level sensors, need to take up glucose for metabolism, a process that produces energy in the form of the universal cellular energy carrier ATP in the mitochondria. Mitochondria are the energy-producing units in the cell. ATP is then transported to the cytosol, the semi-transparant fluid that makes up the majority of the cell content and which encloses other cell compartments. A rise in the ATP:ADP ratio closes a potassium ion channel, causing a change in the electrical voltage potential that is present over the cell membrane. This change opens calcium channels that react to changes in the voltage potential and calcium enters the cell. The influx of calcium triggers the release of insulin from the β-cell. Insulin can bind to insulin receptors, like a key fits into its lock, on its target tissues in order to generate a signal. The main target tissues of insulin are the liver, skeletal muscle and fat tissue. Skeletal muscle and fat tissue respond to insulin by increasing the uptake of glucose from the blood, and the liver responds by decreasing glucose production. Glucagon is released from α-cells, which are sister cells of the β-cells in the endocrine islets in the pancreas. The actions of glucagon work in the opposite direction of insulin. When blood glucose levels fall, glucagon signals to the liver to increase the production of glucose. In skeletal muscle, glucagon promotes the production of glucose from intracellular glycogen stores, providing fuel to the musculature. These actions of glucagon prevent a dramatic fall in blood glucose levels.

In Type 2 Diabetes, the regulation of blood glucose levels by insulin is affected in two ways: the release of insulin from the β-cells in the pancreatic islets is insufficient and the action of insulin on its target tissues is decreased. The latter process is known as insulin resistance. The loss of regulation manifests itself as high levels of blood glucose and this is usually treated first with diet and exercise but can be supplemented with oral drugs to augment insulin secretion and finally with insulin itself. However, the mean blood glucose levels of patients with Diabetes Mellitus are usually higher compared to healthy individuals. This is one of the causes of the development of diabetic complications such as cardiovascular disease, kidney problems, and eye problems. Complications of Diabetes Mellitus develop successively in most patients; in Type 2 Diabetes cardiovascular complications are a major cause of death.

In this thesis, we have attempted to identify mechanisms that are involved in the development of Type 2 Diabetes as well as in the development of complications of Diabetes Mellitus.

In our first study we investigated the role of voltage-sensitive calcium channels in insulin release in rat β-cells. Different types of voltage-sensitive calcium channels exist and their respective contribution to insulin release differs between different species. We determined that one calcium channel in particular, $Ca_V1.2$, is required for insulin release in the rat β-cells that we studied. We compared these β-cells to other related rat β-cells, which do no release insulin in response to glucose and therefore are used a model for β-cell dysfunction, and found that this lack in insulin release is not due to changes in the voltage-gated calcium channels. We suspect that the differences in insulin release between these β-cells are due to differences prior to the opening of the calcium channels.

Next, we studied how the insulin-releasing β-cell adapts to insulin resistance. The mice were made insulin resistant by feeding them a diet with a high fat content. In insulin resistance, higher levels of insulin are required for the maintenance of normal blood glucose levels. Thus, the β-cells must release far more insulin than normally and if they fail to do that, Type 2 Diabetes develops. The mice we studied increase the amount of insulin that they release and do not develop Diabetes Mellitus. We investigated which mechanisms the β-cells use to release more insulin in these mice, preventing the development of diabetes. We found that the β-cells release insulin in response to other fuels than glucose, such as fat but also amino acids (from protein) and that this possibly could be due to the high amount of fat in the β-cell. Fat was accumulating in the β-cells, which was clearly visible as small lipid droplets, using the electron microscope. We think that the high amount of fat in the β-cells disturbs the glucose transporting proteins. In addition, the β-cells of the mice with the high-fat diet displayed a larger mitochondrial area. This might be necessary for the increase in the amount of insulin that was released in order to maintain the blood glucose levels within the boundaries.

The mice strain that we used in the high fat diet study was recently reported to spontaneously develop glucose intolerance, that is, they fail to produce sufficient insulin in response to glucose in a test situation. This glucose intolerance was found to be due to a mutation in a gene involved in the breakdown of glucose and the creation of energy, which signals for the release of insulin. We investigated whether the mice that we used also harbored this mutation but we found that this was not the case. We were unable to detect changes in glucose tolerance in our mice either. We went on to study a related

mouse strain, one frequently used in studies of β-cell specific knockout of genes, for the presence of glucose intolerance. Since these mice often are used to study diabetes-related processes, spontaneous glucose intolerance would negatively affect the conclusions drawn from the knockout of specific genes. However, also this mouse strain was not spontaneously glucose intolerant either.

Finally, we studied insulin action. As mentioned above, insulin exerts its effects on target tissues by binding to the receptor. The insulin receptor is part of a family of related receptors. One member of this family is the insulin-like growth factor-I (IGF-I) receptor. This receptor usually binds IGF-I, which is a hormone that resembles insulin and that is important in growth and development but it also plays a role in control of blood glucose levels. The release of insulin from the β-cell causes the release of IGF-I from the liver where it is produced. IGF-I levels are also disturbed in Diabetes Mellitus. The insulin receptor and the IGF-I receptor have distinct but also overlapping functions. The receptors are very similar in their appearance and hybrid receptors have been found that consist of one half of an insulin receptor and one half of an IGF-I receptor. These hybrid receptors signal to the cell as IGF-I receptors even when the binding of insulin activates them. Since complications of Diabetes Mellitus often result from blood vessel-related problems, we wanted to explore the presence of receptors for insulin but also for IGF-I in human blood vessel cells. We have found that insulin receptors and IGF-I receptors are present on the wall of human blood vessels. IGF-I receptors were found to be more numerous than insulin receptors. These receptors can be activated by their respective ligand (or key) and then send a signal further into the cell. We also have indications of the presence of hybrid receptors, which appear to be activated by IGF-I. This finding indicates the importance of IGF-I in the development of vascular complications in Diabetes Mellitus.

In conclusion, we have studied different aspects of Type 2 Diabetes. None of the findings is in and of itself a target for pharmacological interventions in the treatment of Type 2 Diabetes. Nonetheless, all contribute small pieces to the vast and ever-expanding puzzle of Type 2 Diabetes.

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