Chapter 1

General introduction

Introduction

Type 2 diabetes mellitus is a serious chronic disease, with important consequences for every- day life. Incidence and prevalence are rapidly increasing in the last decennium, not only in the developed countries but even more in the developing countries. At 1 January 2008, 670.000 inhabitants of the Netherlands had type 2 diabetes (± 4%) and another 250.000 adults were unaware that they had this disease (1). It is expected that the amount of diabetic patients in the Netherlands will increase to 1.3 million in 2025. The WHO has estimated that in the year 2000, 171 million adults had type 2 diabetes mellitus and that this will increase to 366 million in the year 2030 (2). To prevent this increase, we need more knowledge of the pathophysiology of type 2 diabetes mellitus and the phenotypes at high risk, in order to develop more effective prevention strategies and to improve treatment possibilities.

Type 2 diabetes mellitus will develop when insulin secretion is not adequate for the prevailing insulin sensitivity. As long as the insulin secretion can keep up with the decreasing insulin sensitivity, there will be no symptoms of glucose intolerance. There is a continuing debate about what comes first; the decreasing insulin sensitivity or the impaired insulin secretion. During each stage of the development of type 2 diabetes mellitus, insulin resistance and insulin secretory dysfunction are independent predictors of worsening glucose tolerance and are, therefore, both targets for the primary prevention of the disease (3). Since better tests were developed to assess the insulin secretion and more research was performed in persons with different degrees of glucose tolerance, small impairments of β-cell function can already be detected in persons without any symptom of hyperglycaemia or type 2 diabetes mellitus (4).

Insulin secretion

β-cells in the pancreas islets are responsible for the insulin secretion. Glucose is the most potent secretagogue as it produces robust insulin secretion in a few minutes after entering the β-cell and the stimulatory effect lasts as long as the plasma glucose is elevated. The β-cell insulin secretory response to glucose occurs in two phases: an acute first phase, lasting a few minutes and then declining followed by a gradually increasing second phase to a peak within 30-40 minutes. Glucose is rapidly transported into the β-cells, largely via the

GLUT1 transporter and partly via the GLUT2 transporter (5;6). Next glucose phosphorylation takes place by glucokinase, a strict glucose specific enzyme, that has demonstrated to be the key regulator of the glucose sensing in β-cells (7;8). The end product of this glucose metabolism, pyruvate, enters the mitochondria, were it follows two different routes. The first route is oxidation to acetyl-CoA, which provides a large amount of ATP. The increased cellular ATP/ADP ratio closes K_{ATP}-sensitive channels, resulting in membrane depolarization followed by Ca^{2+} influx through voltage-gate-dependent Ca^{2+} channels. This causes exocytose of insulin granules. Next to this K_{ATP}- dependent route the mitochondria provide a K_{ATP}- independent way of glucose stimulated insulin secretion by carboxylation of pyruvate to oxaloacetate by the enzyme pyruvate carboxylase. Metabolites produced by the mitochondria are exported to the cytosol and function as intracellular messengers to support insulin secretion. Among these amplifying signals are NADPH, GTP, Malonyl-CoA, Long chain acyl-CoA, Glutamate and PEP (9).

The most important physiologic non-glucose secretagogues that increase the insulin secretion are incretins such as glucose-dependent insulin releasing polypeptide (GIP) and glucagon-like peptide-1(GLP-1). Immediately after oral ingestion of nutrients GIP is mainly secreted by the K cells in the upper small intestine while GLP-1 is predominantly secreted by entero-endocrine L cells located in the distal intestine (10-12). This prompt release is probably more indirect controlled by neural and endocrine factors in the proximal gastrointestinal tract, while later incretin secretion is maintained by arrival of nutrients lower in the intestine. Binding of GIP and GLP-1 to their specific receptor at the β-cell membrane causes the activation of adenyl cyclase via the G protein and leads to an increase of intracellular cyclic adenosine monophosphate (cAMP). This evokes a cascade of intracellular events resulting in increased concentration of cytosolic Ca^{2+} which drives the exocytose of insulin granules. Incretin receptors are expressed in many other tissues including several brain areas and the heart. Besides the enhancement of insulin secretion, both incretins promote β-cells proliferation while GLP-1 also stimulates insulin biosynthesis, reduces food intake, inhibits glucagon secretion and decreases gastrointestinal secretion and motility.

Amino acids and fatty acids stimulate insulin secretion not only by enhancing the incretin production in the intestinal cells (10;13), they have also a specific effect on β-cells. Charged amino-acids like lysine and arginine cross the β-cell membrane via a transport system specific for cationic amino acids. The accumulation of the positive charged

molecules directly depolarizes the β-cell membrane leading to calcium influx and consequently increased insulin secretion (14;15). On the other hand, fatty acids play a role in the intracellular amplification pathway of insulin secretion (15) and may remodel the plasma membrane to facilitate insulin secretion (9).

Age appears to be negatively correlated with β-cell function in glucose tolerant Caucasians, even after correction for insulin sensitivity and this might be due to an impairment in proinsulin conversion to insulin (16;17).

The autonomic regulation of the β-cell function is influenced by the splanchnic nerve of the sympathetic nervous system (SNS) and the vagus nerve of the parasympathetic nervous system (PNS) (18). The splanchnic nerve releases norepinephrine from nerve terminals and epinephrine from the adrenal medulla, initiating catabolic metabolic processes including inhibition of insulin secretion. In contrast the vagus nerve mediates anabolic responses to internal stimuli from the viscera and external stimuli from the sensory components of food. Activation of the vagal efferent activity occurs at the onset of and during meal ingestion and plays an important role in the acute and further postprandial insulin responses. The well-known neurotransmitter of the vagus nerve, acetylcholine, acts on the β-cells by muscarine receptors. Consequently, a combination of reactions follows, including increment of cytosolic concentration of Ca^{2+}, independently of extracellular Ca^{2+} uptake, stimulation of the formation of arachidonic acid and activation of protein kinase C, resulting in a rapid stimulation of exocytosis and insulin secretion (19). There are three more neurotransmitters localized to islet parasympathetic nerves: vasoactive intestinal polypeptide, gastrin releasing peptide and pituitary adenylate cyclase activating polypeptide. They all stimulate insulin secretion by activating intracellular signalling mechanisms, which are partially different. Prolonged mild hyperglycaemia results in a compensatory increase in insulin secretion, which is partially mediated by an induction in vagal efferent activity (20).

Autonomic and endocrine responses to food consumption, which are evoked by sensory mechanisms before nutrients have been absorbed, are called ‘cephalic phase responses’ (19). The insulin secretion in the first 3-4 minutes of a meal intake is the result of three successive pathways: first, the afferent pathway activated by olfactory, visual, gustatory and oropharyngeal mechanical receptors, secondly the integration of these stimuli in the brain and finally the efferent pathway, mediated by the cholinergic neurons. Although the contribution of the cephalic phase to the entire postprandial insulin secretion

is only 1-3%, the cephalic phase is probably of considerable functional importance for glucose tolerance after meal intake. Inhibition of the early (15 min) response to a meal by the ganglionic antagonist , trimetophane, is accompanied by increased post prandial glucose concentrations at min 45 and 60 (19).

Increased sympathetic activity results in inhibition of glucose stimulated insulin secretion in situations of stress, including exercise and trauma (21). The neurotransmitter norepinephrine activates the α_2- adrenergic receptors in the β-cell membrane. The inhibition of insulin secretion is mediated by hyperpolarisation of the β-cells through opening of the ATP- regulated K^+ channels. This inhibits the Ca^{2+} uptake and reduces the cytosolic concentration of Ca^{2+}. Reduced formation of cyclic AMP and inhibition of the metabolic processes leading to exocytosis have also been shown as cause of reduced insulin secretion after activation of α_2- adrenergic receptors in the β-cell membrane. Although norepinephrine can also stimulate insulin secretion by activation of the β_2-adrenergic receptors on the β-cell membrane, resulting in increased formation of cAMP in the β-cells (19), increased sympathetic activity results predominantly in decreased insulin secretion (21).

Besides the parasympathetic and sympathetic nervous systems, each individual islet is also extensively innervated by a network of sensory nerves and by nerve fibres, stained for a marker of nitric oxide synthethase. However the role of these two types of nerve fibres is far from understood (19).

Insulin secretion in adult life may also be related to pre-natal circumstances. The consequences of the famine during the Dutch Hunger Winter of 1944-1945 have been extensively investigated. It appeared that foetal malnutrition especially during the first 6 months gives rise to impaired glucose tolerance in adult life based on an insulin secretion defect (22).

Insulin signalling and insulin action

The insulin molecule consists of two polypeptide chains, the A chain (21 amino acids) and the B chain (30 amino acids), linked by two disulphide bridges. The insulin cell-surface receptor is a heterotetrameric receptor, composed of two extracellular α subunits and two β subunits that contain an extracellular portion, a transmembrane domain and an intracellular part. Insulin binding to the α subunit results in phosphorylation and activation of the

tyrosine kinase in the intracellular part of the β subunits (23). This activates the insulin receptor substrate (IRS) proteins 1-4, which are the main mediators of the intracellular insulin receptor signalling events (24). The four IRS proteins are tissue specific; IRS-1 protein mediates the insulin action specifically in the skeletal muscle, while IRS-2 protein acts in the liver. The tyrosine phosphorylation of IRS-1 leads to two major signalling pathways e.g. the phosphatidylinositol-3'-kinase (P13K) pathway and the mitogen-activated protein kinase (MAPK) pathway. The P13K pathway plays a crucial role in the metabolic actions of insulin, by stimulating glycogen, lipid and protein synthesis. It also stimulates nitric oxide production a potent vasodilator and anti-atherogenic agent. In muscle and fat cells this pathway also affects the insulin regulated glucose transport (GLUT4) system, which facilitates the rapid uptake of glucose through the cell membrane (23). The activation of MAPK pathway leads to intra-nuclear processes, which influence transcription factors and DNA synthesis. This results not only in cell growth, cell proliferation and cell differentiation, but also in activation of multiple inflammation pathways (25). In short, the core business of insulin in the body is energy storage.

Insulin sensitivity is at the physiological level associated with obesity, physical inactivity and aging. Decreased insulin sensitivity is characterized by an impaired ability of insulin to inhibit hepatic glucose production and to stimulate glucose uptake by skeletal muscle. Insulin also fails to suppress lipolysis in adipose tissue. The molecular mechanisms underlying a decrease in insulin sensitivity are not all precisely known, but may be mainly based on a deregulation of one of the many steps of the insulin signalling pathway. Protein tyrosine phosphatises (PTPs), which dephosphorylate the insulin receptor or downstream substrates may be key regulators of the insulin receptor signal transduction pathway and for the most part attenuate insulin action (26). Recent studies in human skeletal muscle of insulin resistant type 2 diabetic and obese non diabetic individuals showed profound insulin resistance in the P13K pathway with normal stimulatory effect of insulin on the MAPK pathway (25). This defect in insulin signalling impairs not only glucose uptake, glucose metabolism in the muscle cells and NOS synthesis but, because of the persistent hyperinsulinaemia, at the same time activates via the MAPK pathway multiple genes coding for pro–inflammatory .mediators (TNFα, IL-1B, PKC). These pro-inflammatory mediators inhibit the intracellular insulin signalling and induce the degradation of IRS-1 by phosphorylation of the Serine residues on the IRS proteins (27).

Insulin signalling can also be inhibited by signals from other pathways, like that in lipotoxicity. Obesity is often characterised by a state of low grade chronic inflammation with increased levels of pro-inflammatory cytokines and their effects on insulin resistance by serine phosphorylation of IRS-1(25). Studies have shown that fat accumulation in muscle and hepatic cells are correlated with organ-specific insulin resistance. Increased release of free fatty acids from the adipose tissue decreases insulin mediated glucose transport in skeletal muscle and impairs suppression of glucose production by the liver(27). Adipocytes and infiltrated macrophages of visceral fat of obese and type 2 diabetic individuals secrete pro-inflammatory cytokines (TNFα, Interleukin-6), acute phase reactants (C-reactive protein) and hormones (leptin and resistin) which also induce insulin resistance. Moreover, visceral adiposity is a state with a relative deficiency of adiponectin, a potent insulin-sensitizing hormone (24).

The importance of insulin sensitivity and specially the role of the adipose tissue in the development of diabetes mellitus has recently been shown by the results of the CANOE (Canadian Normoglycemia Outcomes Evaluation) trial (28). A low dose combination therapy of rosiglitazone (a PPARγ agonist that increases insulin sensitivity among others by its action on adipose tissue and fatty acids in the muscle) with metformin (a biguanide that reduces hepatic glucose production and increases the peripheral insulin sensitivity) appeared to be highly effective in the prevention of type 2 diabetes in patients with impaired glucose tolerance. The low dose combination therapy did not only results in a smaller decline of insulin sensitivity but also in a reduction in inflammation and improvement in hepatic function.

Insulin sensitivity declines slowly during aging, but this may be due to age–related changes in body composition, rather than a consequence of aging itself (29). Increased insulin resistance in elderly was found to be associated with fat accumulation in muscle and liver cells that may be a result of age-associated decrease in ATP production by the mitochondria (30). However, a recent study of Karakelides (31) showed that an age related decrease in muscle mitochondrial function was neither related to adiposity nor insulin sensitivity.

Genetic and environmental factors

The importance of genetic influences is sustained by twin studies, and a strong familial aggregation. In 1981 Barnett (32) showed a nearly complete concordance rate for type 2 diabetes mellitus in identical twins while in the few discordant pairs the unaffected twin already showed metabolic abnormalities. His conclusion that genetic factors are predominant in the aetiology of type 2 diabetes mellitus has been confirmed by many twin (33-52) and family (53-63) studies in the following decades. A positive family history immediately increases the chance to get the disease. The risk is six times higher when two first degree relatives have type 2 diabetes mellitus and at least two times when one first degree relative is affected (64;65). Further evidence for a genetic role is the wide variation in prevalence among different ethnic groups (66;67).

At the end of the 20th century twin and family studies also started to estimate the heritability of individual differences in glucose and insulin levels. Most of these studies were performed with only fasting glucose and insulin levels, but a few studies have also addressed heritability of the responses to glucose challenge tests like the Oral Glucose Tolerance tests, the intravenous glucose tolerance test (mainly for assessment of β-cell function) and the euglycaemic-hyperinsulinaemic clamp test (for insulin sensitivity only). Table 1.1 and 1.2 give an overview of twin and family studies, performed from 1996 till 2010, that assessed the heritability of insulin sensitivity and insulin response in many different ways. Table 1.3 summarizes the results from studies, performed in the same period, that estimated heritability for clinical indicators of (pre)diabetic state.

A further step towards a better understanding of the genetic variation involved in type 2 diabetes mellitus was the identification of the actual genetic variants. In the last decade studies came out that tested the association of variants in candidate genes with measures of glucose metabolism and/or the risk of type 2 diabetes mellitus. But increasingly the candidate gene approach has given way to the genome wide association (GWAS) approach. Large collaborative consortia across many different research groups like MAGIC (the Meta-Analyses of Glucose an Insulin related Traits Consortium) made it possible to combine the data of tens of thousands of subjects to identify new genetic variants that affect glucose metabolism and/or the risk of type 2 diabetes mellitus. So far, GWA studies have uncovered 26 confirmed gene variants that are associated with a higher risk for the development of type 2 diabetes mellitus (68;69) and at least fifteen of these

Table 1.1: Heritability estimates of insulin resistance and insulin sensitivity from studies between 1996 and 2010

Test	reference no	formula	Heritability %	covariate
Insulin RESISTANCE				
Fasting blood levels	(38;53;63)	HOMA-IR= (fasting insulin mU/l x fasting glucose mmol/l)/22.5	22 – 42 - 48	A, S
	(50;56)	HOMA-IR= (fasting insulin mU/l x fasting glucose mmol/l)/22.5	23 - 48	A, S, BMI
	(61;71)	HOMA-IR= (fasting insulin mU/l x fasting glucose mmol/l)/22.5	8 - 38	A, S, E
	(37)	Log (HOMA-IR)	8	
	(54)	Log (HOMA-IR)	12	
	(54)	Log (HOMA-IR)	16	A, S, BMI
	(37)	HOMA-R'=[(log fasting insulin)-c] x fasting glucose	59	
	(36)	HOMA2-IR computer model	58	A, BMI, W
Insulin SENSITIVITY				
Fasting blood levels	(57)	ISI 0= Insulin Sensitivity Index 0 =	37	A, S
			28	A, S, BMI
	(60)	HOMA-%S	46	A, S, BMI
	(46)	Basal glucose uptake in μmol/l $kgFFM^{-1}$ min^{-1}	Young 27	S
			Old 67	S
Oral glucose tolerance test (OGTT)	(57)	ISI 120 = 10^4 / Insulin 120 minute x glucose 120 minute	34	A, S
			32	A, S, BMI
FSIVGTT+insulin	(54)	Log(S1+1)	29	
			33	A, S, BMI
FSIVGTT+ tolbutamide	(55)	Si = insulin sensitivity index	38	
	(62)	Si = insulin sensitivity index	28	A, S, BMI
FSIVGTT	(59)	Si MINMOD analysis computer program	44	A, S, BMI
Euglycaemic hyperinsulinaemic Clamp	(61)	M (lbm)= glucose disposal in mg $kglbm^{-1}$ min^{-1}	24	A, S, E
	(39;46)	Insulin stimulated glucose uptake in μmol/l $kgFFM^{-1}.min^{-1}$.	37 - 55	S

FSIVGTT = frequently sampled intravenous glucose tolerance test; HOMA-IR = Homeostasis Model Assessment of insulin resistance; HOMA-%S = Homeostasis Model Assessment of insulin sensitivity, computer model; ISI = insulin sensitivity index; c = a constant derived from regression analysis of Ln(Ins0) *vs* Glucose 0; S_1 = insulin sensitivity index, based on minimal model of Bergman, ref (54); A = age; S = sex; BMI = Body mass index; E = ethnicity.

Table 1.2: Heritability estimates of insulin levels c.q.insulin response with respect to glucose level as surrogate measure of β-cell function

Test	1st Author	Reference no	Formula	Heritability %	covariates
Fasting blood levels	Katoh	(38)	HOMA- β = (20xIns0(mU/l)/Gluc0(mmol/l-3.5)	38	A, S
	Souren	(50)	HOMA- β	62	A, S, BMI
	Falci	(36)	HOMA2-%B	63	A, BMI
	Jenkins	(37)	HOMA- β'= (Ln(Ins0) – c)/Gluc0	68	women
	idem		Log (HOMA- β)	28	women
	Mills	(60)	HOMA%-B (computer model)	78	A, S, BMI
OGTT	Hanson	(57)	CIR_{120}=Ins $_{120}$/G_{120}x(G_{120}-70mgr/dl)	24	A, S, BMI
OGTT insulin secretion	Lehtovirta	(40)	OGTT β index computer model	53	
FSIVGTT+ tolbutamide	Elbein	(55)	Acute Insulin Response (2-10)	38	
	Hong	(59)	Acute Insulin Response (2-10)	46	A, S, BMI
	Lehtovirta	(39)	Acute Insulin Response (1-10)	55	S
			Acute Insulin Response (1-10), adjusted for isgu	41	S
	Lehtovirta	(39)	Acute Insulin Response (10-60)	58	S
	Lehtovirta	(40)	Readily Releasable Insulin	76	S

HOMA-β = Homeostasis Model Assessment of β-cell activity; A = age; S = sex; BMI = Body mass index; c = a constant derived from regression analysis of Ln(Ins0) *vs* Gluc0; HOMA2-%B = software (http://www.dtu.ox.ac.uk/homa) was used. OGTT = oral glucose tolerance test; OGTT β index: β-cell ability to increase insulin secretion in response to glucose; FSIVGTT = frequently sampled intra venous glucose tolerance test; Readily Releasable Insulin = the first peaking phase of insulin secretion during IVGTT, computer model. isgu = Insulin stimulated glucose uptake

Table 1.3: Range of Heritability estimates for clinical indicators of (pre)diabetic state in 27 twin and family studies

Test	Fasting glucose	OGTT Glu 120	Fasting insulin	OGTT Ins 120	HbA1c	D.I.	BMI
Heritabilities in % in twin studies	12 - 75	35 - 62	14 - 54	28 - 51	62	75 - 84	50 - 90
Reference no	(33;37-39;41-44;47;48;50)	(33;38;41;44;45;47)	(33;34;38;39;41-45;47;48;50;52)	(41)	(49)	(46)	(33;34;38;43-45;50;72)
Heritabilities in % family studies	7 - 77	17	8 - 51	35	55 - 60	23 - 67	20 - 80
Reference no	(53;56;58;60;61;63)	(53)	(53;54;57;58;61)	(57)	(60;63)	(55;59)	(44;53;58;61;63;72)

OGTT Glu 120 = Glucose concentration at 120 minutes during Oral Glucose Tolerance Test; OGTT Ins 120 = Insulin concentration at 120 minutes during Oral Glucose Tolerance Test; D.I.= Disposition index (Insulin secretion x insulin sensitivity); BMI = Body mass index.

genes affect β-cell function. A number of genetic loci have also been revealed for glucose and insulin metabolism as reviewed by Ingelsson (70). Nearly all these loci derive from studies that performed glucose and insulin measurements in the fasting state or during an OGTT.

Outline of the thesis

Despite impressive progress still much of the pathophysiology of type 2 diabetes mellitus is unknown. In part this reflects a poor understanding of the causes of interindividual differences in insulin production, even in healthy individuals. The twin-family study presented in this thesis focuses on the function of the healthy β-cell. Its aim is to reveal the genetic and environmental contribution to individual variation in different aspects of β-cell function and to associate the heritable aspects of β-cell function with candidate genotypes arising from ongoing GWA studies.

Chapter 2 details the design of the study, including the recruitment of the participants and a description of the tests of the β-cell function performed. In Chapter 3 we estimate the heritability of the main diagnostic parameters used in type 2 diabetes mellitus, fasting glucose and HbA1c, with special attention to a possible overlap in the genetic influences on these parameters. In chapter 4 the heritability of classical and mathematical model derived β-cell function parameters is estimated during a highly naturalistic challenge, the mixed meal test. This test includes the influence of incretins on the insulin secretion. In chapter 5 we present the use of the extended hyperglycaemic clamp to assess the heritability of insulin secretion after different intravenous secretagogues. A euglycaemic-hyperinsulinaemic clamp was performed in the same subjects to estimate the heritability of insulin sensitivity. Associations between selected genotypic variants from recent GWA studies and β-cell function are described in the last two chapters. Chapter 6 shows the association between eight type 2 diabetes mellitus related gene variants and the insulin response, stimulated by the three different secretagogues during hyperglycaemic clamps. To increase the power of this investigation, four different clamp studies were combined. In Chapter 7 we show that variation in several type 2 diabetes mellitus risk genes is associated with different aspects of β-cell function, assessed with the extended hyperglycaemic clamp tests.

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