Pancreatic β- and α-cell adaptation in response to metabolic changes

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Johanne Hendrike (Rianne) Ellenbroek

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Chapter 1

General introduction

Diabetes mellitus

In the second century AD, a Greek physician called the disease that was characterized by extreme loss of urine 'diabetes', meaning siphon. In 1679, the physician Thomas Willis added the word 'mellitus' to diabetes, referring to the sweet taste of the urine (1). Diabetes mellitus is a disease of metabolic dysregulation, in particular dysregulation of glucose metabolism. In healthy individuals, blood glucose concentrations are tightly balanced by two counteracting hormones, insulin and glucagon, that are secreted by β- and α-cells, respectively, which are endocrine cells located in the pancreas (Fig. 1). Diabetes is characterized by an impaired glucose homeostasis due to an absolute or relative deficiency of insulin. Symptoms include polyuria, polydipsia, polyphagia, and weight loss. The diagnosis of diabetes is based on one of the following criteria: percentages of glycated hemoglobulin (HbA1c) ≥ 6.5%, fasting plasma glucose (FPG) ≥ 7 mmol/l, or a 2-hour plasma glucose ≥ 11.1 mmol/l after a 75-g oral glucose tolerance test (2).

In 2013, 382 million people had diabetes worldwide and by 2035 this is expected to increase to 592 million people (3). In The Netherlands approximately 1 million people are diagnosed with diabetes (4). The two major forms of diabetes are type 1 and type 2, although diabetes can also manifest during pregnancy and under other conditions including drug or chemical toxicity and genetic disorders. In type 1 diabetes (~10% of patients) the majority of β-cells is lost due to autoimmune destruction. A combination of genetic and environmental factors activates an immune response against β-cells. In type 2 diabetes (~85% of patients) β-cell function slowly decreases over time and is associated with a loss of β-cell mass up to 65% (5) probably due to metabolic and/or inflammatory factors.

Patients with type 1 diabetes require life-long insulin replacement therapy by multiple-dose insulin or insulin pump therapy. In patients with type 2 diabetes, initially lifestyle changes as healthy eating, weight control and increased physical activity are stimulated to obtain an HbA1c <7.0%. If this target is not met, pharmacological therapy can be initiated, starting with metformin therapy that improves insulin sensitivity and suppresses glucose production by the liver. Many patients will ultimately require insulin therapy due to the progressive nature of the disease (2).

Despite intensive treatment with diets, antihyperglycemic oral agents or insulin injections, normalization of glycemic control can often not be achieved. Patients are at risk to develop acute and long-term complications. Acute complications include diabetic ketoacidosis from persistent hyperglycemia, and hypoglycemic events. Long-term complications include a wide range of microvascular complications such as retinopathy, nephropathy and neuropathy. Furthermore, patients with diabetes have an increased risk for cardiovascular and cerebrovascular disease (6). Therefore, therapies that restore, maintain or prevent loss of functional β-cells are needed for all types of diabetes. For that reason it is critical to better understand mechanisms that regulate β-cell mass growth and function.

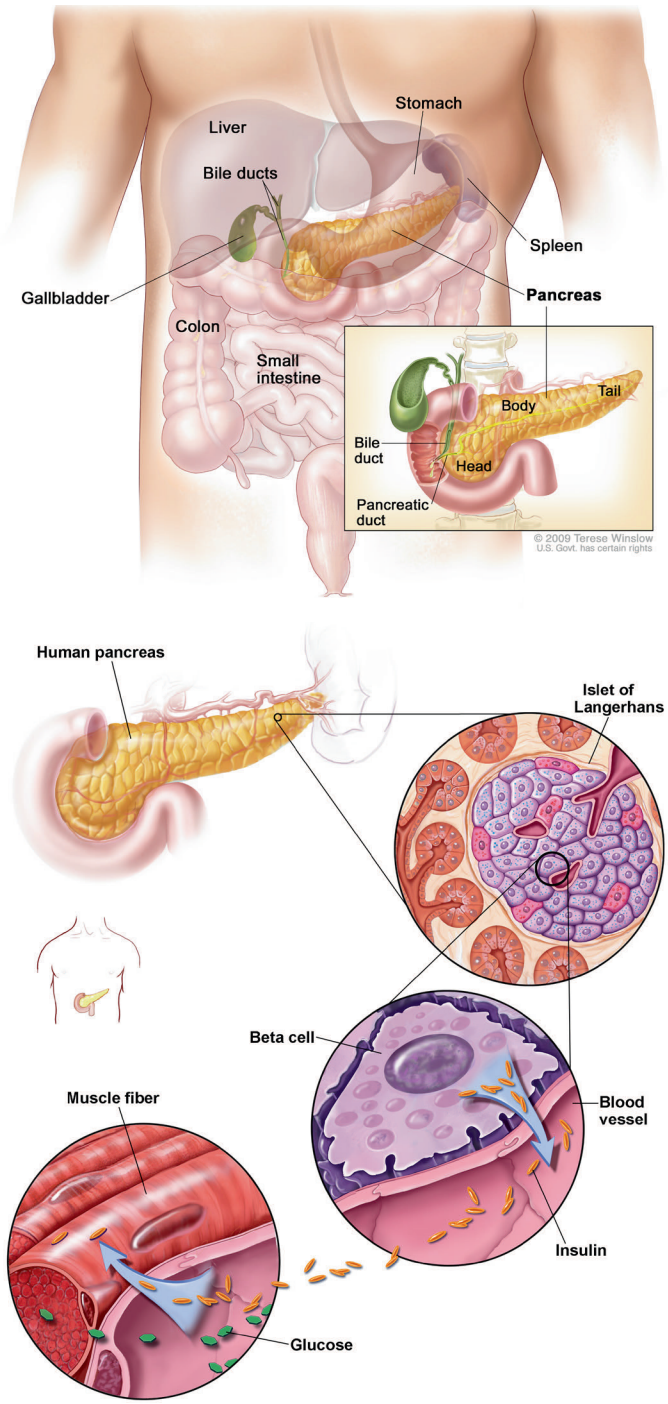

Figure 1. A. Illustration of the pancreas and nearby organs. Inset: Illustration of the head, body and tail of the pancreas. B. Illustration of the insulin-secreting β-cell. *Reprinted with permission from Terese Winslow.*

Glucose metabolism

The blood glucose concentration is determined by the rate of glucose entering the circulation balanced by the removal of glucose out of the circulation. The primary action of insulin is to remove glucose from the circulation whereas its counterpart glucagon stimulates the entering of glucose into the circulation (Fig. 1B). In a fed state β-cells are triggered to secrete insulin, which facilitates the uptake of glucose from blood into cells. Inside the cell glucose will be metabolized via glycolysis, a multistep process of which pyruvate is the end product. Under aerobic conditions, pyruvate enters the citric acid cycle in the mitochondrion resulting in the production of NADH and $FADH_2$ that are subsequently oxidized in the respiratory chain to generate energy (7). Excessive glucose can be stored in the form of the polymer glycogen in the liver and muscle. Also, insulin promotes lipogenesis and protein synthesis and inhibits the oxidation of free fatty acids and protein breakdown (8). During fasting, blood glucose concentrations are increased by the hormone glucagon that stimulates glucogenolysis from glycogen stores and gluconeogenesis from non-carbohydrate sources (9). Furthermore, it counteracts insulin by restraining the synthesis of glycogen, glycolysis and lipid storage. If fasting continues for several days, glucagon can stimulate lipolysis of adipose tissue and proteolysis from muscle tissue of which the substrates can be used to generate glucose by gluconeogenesis in the liver (10). At the same time, glucagon can stimulate ketogenesis, providing ketone bodies that can be used as an alternative fuel for the brain when glucose is sparse (9).

β-Cells

The islets of Langerhans are clusters of endocrine cells that are scattered throughout the exocrine pancreas. Islets represent only 1 to 2% of the pancreas. The islets are composed of insulin-producing β-cells (60-70%), glucagon-producing α-cells (20-30%), somatostatin-producing δ-cells, pancreatic polypeptide (PP) producing PP cells and ε-cells that produce ghrelin (11, 12). The remainder of the pancreas consists of exocrine cells that secrete digestive enzymes that are transported to the duodenum via the pancreatic duct system. Pancreatic islets are highly vascularized to enable efficient secretion of hormones into the circulation and densely innervated allowing control of the glucose homeostasis by the autonomic nervous system (13).
The gene for insulin is located on chromosome 11 in humans and its product is a 110-amino acid precursor peptide called preproinsulin. The signal peptide of the protein brings preproinsulin into the lumen of the rough endoplasmic reticulum (ER) where it is removed generating proinsulin. Within the ER, proinsulin folds into a three-dimensional structure and is brought via vesicular transfer to the Golgi apparatus. In the Golgi, proinsulin enters immature secretory vesicles and is cleaved by prohormone convertases generating insulin and C-peptide. Insulin and C-peptide

are stored in secretory granules together with islet amyloid polypeptide. These insulin granules accumulate in the cytoplasm of the β-cell awaiting a signal to be released from the β-cell (14, 15). The insulin secretory pathway becomes activated when glucose enters the cell through the glucose transporter-2. Oxidative metabolism of glucose leads to an increase in the ATP/ADP ratio. This leads to closure of ATP-dependent potassium channels. The subsequent membrane depolarization results in an influx of Ca^{2+} through the opening of voltage-gated calcium-channels. The increase in intracellular calcium concentration stimulates the fusion of insulin granules to the cell membrane and exocytosis of insulin (16, 17).

Embryonic development and topological heterogeneity of islets in the pancreas

During embryonic development the pancreas originates from two epithelial buds, a ventral and dorsal bud, which protrude from the embryonic gut epithelium and converge to form the definitive pancreas (Fig. 2). The developing pancreatic duct epithelium consists of multipotent pancreatic progenitor cells that will give rise to all mature pancreatic cell types and undergoes extensive branching into a highly organized tubular network. Within the ductal epithelium endocrine progenitor cells arise, characterized by expression of the transcription factors *Pdx1* and *Ngn3*, which delaminate from the duct and migrate into the surrounding mesenchyme and aggregate into cell clusters that ultimately form the islets of Langerhans (18–21). The highest increase in endocrine tissue during development occurs in the second and third trimester of pregnancy in humans. During this period β-cell proliferation is relatively low and data suggest that the majority of new islet cells arise from precursor cells, a process called neogenesis (20, 22). The ventral bud gives rise to the posterior part of the mature pancreatic head and uncinate process, and the dorsal bud forms the anterior part of the head, the body and tail (Fig. 1A). A systematic study of the rat pancreas revealed that in the lower part of the head of the pancreas contains islets with a high percentage of PP-cells, whereas few glucagon-positive cells are observed in this same region (23). In humans, similar PP-rich lobules are identified in the pancreatic head region, most likely the part originating from the ventral pancreatic bud during embryogenesis (24–26). Also, islets derived from the dorsal bud secrete more insulin after a glucose stimulus compared to islets derived from the ventral bud in rats (27). Furthermore, in adult human pancreas the density of islets is higher in the tail-region compared to the head and body-region of the pancreas (28–30). Altogether this shows that islet morphology and function can be different throughout the pancreas.

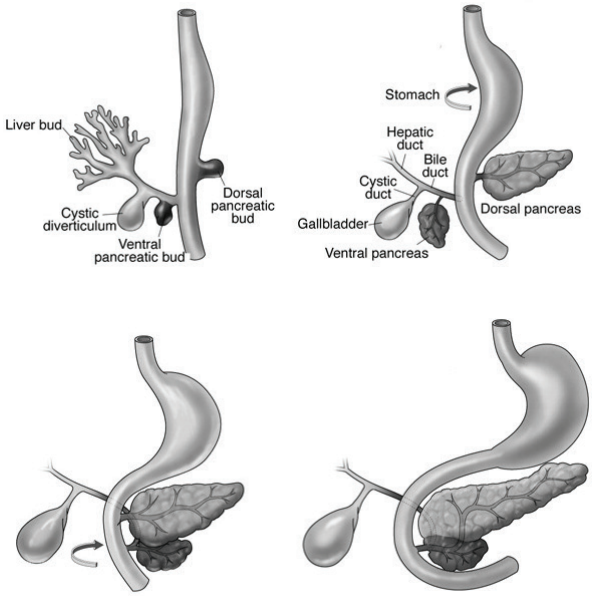

Figure 2. Organogenesis of the pancreas. The pancreas originates from 2 buds, the ventral and dorsal pancreatic bud. The ventral and dorsal pancreas fuse to form the mature pancreas. *Adapted with permission from Cano et al. Gastroenterology 2007* (19).

β-Cell mass adaptation

In adults the β-cell mass is tightly controlled in order to maintain blood glucose concentrations within a narrow range. When the demand for insulin is chronically increased by physiological or pathological changes, such as obesity, pregnancy, glucocorticoid treatment and pancreatic damage, β-cells can adapt by enhancing insulin secretion via increased β-cell function and/or increased β-cell mass (Fig. 3). Inadequate β-cell adaptation leads to the development of hyperglycemia and eventually diabetes mellitus (28, 31).

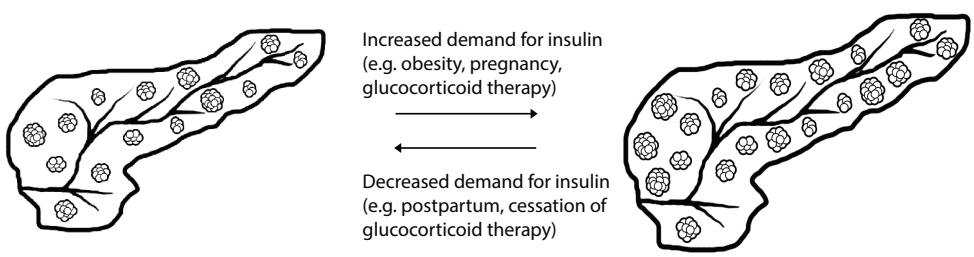

Figure 3. Changes in the demand for insulin are associated with an adaptation of the β-cell mass.

Obesity

Obesity in humans is associated with an increased insulin secretory response following a glucose or meal challenge (32, 33). In 1933 Ogilvie reported that islets in humans with obesity were enlarged (34). Later this observation was confirmed by several studies comparing β-cell mass between non-diabetic obese and lean individuals. These studies have reported increases in β-cell mass ranging from 20 – 100% in obese subjects (28, 31, 35, 36). Recently, Saisho et al. (37) studied the largest population of pancreas donors. A 50% increase in β-cell mass was found when comparing 61 obese vs. 53 lean subjects. Obesity is associated with insulin resistance, which results in an increased demand for insulin from β-cells (16). Mice fed a high-fat diet become obese and develop insulin resistance that is associated with an increased β-cell mass (38, 39). In young (5 - 6 weeks old) mice the β-cell mass increase ranges from 2 – 3.5 fold after a high-fat diet for 8 weeks (39, 40). Also, genetic mutations in rodents that lead to obesity and insulin resistance, such as leptin-deficient *ob/ob* mice and *db/db* mice or Zucker *fa/fa* rats that have a defective leptin-receptor, are associated with a compensatory increase of the β-cell mass (41–44). The development of diabetes in *Macaca mulatta* is associated with obesity and insulin resistance and in normoglycemic, hyperinsulinemic monkeys associated with an increased β-cell area (45).

The importance of insulin resistance generating an environment of increased insulin demand is illustrated by mice that are double heterozygous for null alleles in the insulin receptor (IR) and insulin receptor substrate-1 (IRS-1) genes. These mice develop severe insulin resistance that is associated with a massive increase of the β-cell mass, up to 30-fold (46). Specific knockout of the insulin receptor in the liver (LIRKO) also results in dramatic insulin resistance and is associated with a 6-fold increase in β-cell mass (47). Mezza et al. (48) reported an increased islet size in insulin resistant non-diabetic humans, that was inversely correlated with insulin sensitivity. Recently it was shown that transplantation of human islets isolated from non-obese donors into insulin resistant mice results in β-cell mass adaptation of the human islet graft *in vivo* (49).

Together these studies show that in obesity-related, diet- or genetically-induced insulin resistance there is a compensatory growth of the insulin producing β-cell mass. Since about 80% of obese humans remain non-diabetic (50), this adaptation of the β-cell mass is successful in most cases.

Pregnancy

Pregnancy is accompanied with series of metabolic changes including a progressive development of insulin resistance that requires adaptation of the β-cells to maintain normoglycemia (51). Several studies have reported increases of the islet mass during pregnancy in rodents (52). The extent of the increase varies from 1.5- to 2-fold (53–55). Interestingly, postpartum the β-cell mass involuted to prepartum levels (53, 55).

Obviously the possibility to study pancreas material from pregnant women is limited and until today only a few groups have studied the β-cell mass in pregnant women. Van Assche et al. reported an enlargement of the islets of Langerhans and hyperplasia of β-cells resulting in a

2.4-fold increase of β-cell area when comparing 5 pregnant with non-pregnant women (56). More recently, Butler et al. (57) reported a 1.4-fold increase of the β-cell area in the pancreas of 18 pregnant women. Interestingly, this latter group also studied β-cell area in a few pancreas samples from post-partum women and observed a strong tendency for a decrease in β-cell area compared with pregnant women suggesting that, similar to rodents, the β-cell mass returns to baseline levels postpartum. It is thought that failure to compensate β-cells during pregnancy may contribute to the development of gestational diabetes in women, which is a risk indicator for the development of type 2 diabetes later in life (58). Together these studies show that during pregnancy the β-cell mass increases to compensate for the increased insulin demand, a compensation that seems to be reversible postpartum.

Glucocorticoid therapy

Glucocorticoids are essential to the adaptation of the body to fasting, injury, and stress. Their receptors are expressed on most cells, by which they can influence a variety of physiological processes. Therefore, glucocorticoids are used extensively as therapeutic agents, especially for their anti-inflammatory actions. However, patients become glucose intolerant and they have an increased risk to develop diabetes because glucocorticoids antagonize the action of insulin (59). This change in glucose metabolism increases the demand for insulin. In rodents and non-human primates glucocorticoid treatment induces insulin resistance, which is associated with increased insulin secretion and compensatory β-cell mass growth (60–64). Interestingly, discontinuation of the therapy in rats resulted in involution of the β-cell mass to pretreatment levels (64).

Pancreatic damage

When (part of) the endogenous β-cell mass is removed surgically or by physical or chemical damage to the pancreas, an increased demand for insulin on remaining β-cells arises. Multiple animal models have been developed to study the response of the β-cell mass in situations of pancreatic damage. β-Cells can be destroyed using cytotoxic agents, such as streptozotocin (STZ) or alloxan. Following STZ treatment and in the presence of insulin treatment, the β-cell mass in mice regenerated to about 50% after 6 days (65). In STZ-treated newborn rats the β-cell mass was regenerated to 39% of the normal value by day 20 (66). However, no regeneration of the β-cell mass was observed in male vervet monkeys treated with STZ (67). Also removal of 50 – 90% of the pancreas is associated with a (partial) regeneration of the pancreas after a couple of weeks (68–70). So, depending on the treatment regimen, chemically induced destruction or (partial) removal of the β-cells can lead to regeneration of the β-cell mass in rodents (68).
In humans, glucose concentrations begin to rise when the β-cell mass is reduced by approximately 50% (71), which fits the observation that in patients with type 2 diabetes β-cell mass is reduced to 65% compared to non-diabetic subjects (5). No compensatory growth of the β-cell mass was observed in patients that underwent a 50% pancreatectomy because of chronic pancreatitis or

pancreatic cancer (71). Also, hemipancreatectomy in healthy donors resulted in deterioration of insulin secretion and glucose intolerance in 25% of the donors one year after surgery (72). Of 22 children diagnosed with nesidioblastosis who had undergone 90-95% pancreatectomy, 55% showed complete pancreatic regeneration (assessed by ultra-sound, no histology data available) resulting in a pancreas normal in size for the age (73). Since these patients were normoglycemic, this suggests that the regenerated pancreas consisted of an adequate number of endocrine cells. Altogether this shows that, in contrast to rodents, there is no evidence in adult humans that the β-cell mass regenerates in response to pancreatic damage.

Mechanisms of β-cell mass adaptation

The β-cell mass is determined by the balance of β-cell renewal and loss. Mechanisms potentially involved in β-cell mass regulation are proliferation and apoptosis of existing β-cells and the formation of new β-cells from precursor cells (neogenesis). More recently it was reported that mature cells (e.g. α-cells or acinar cells) are able to transdifferentiate into insulin-producing cells (74, 75).

β-Cell proliferation

In young mice (5 - 6 weeks) basal β-cell proliferation detected by Ki67 staining is ~2.5% (76, 77). β-Cell proliferation is restricted with advanced aging and drops to 0.1 – 0.3% in older (> 1 year) mice (38, 77). β-Cell mass adaptation or regeneration in response to diet-induced obesity, pregnancy, glucocorticoid-induced insulin resistance and pancreatic damage have all been associated with increased number of proliferating β-cells in rodents. β-Cell proliferation was found to be increased from ~0.6% to 4.5% Ki67 positive β-cells after a high-fat diet for 8 weeks (40). Recently, Stamateris et al. (78) showed that β-cell proliferation already increases within the first week of high-fat diet feeding. In rodents and non-human primates, glucocorticoid treatment increases β-cell proliferation (60, 63, 79). In pregnant rodents, β-cell proliferation increases until mid gestation and then declines to prepartum levels (53, 55, 80). DNA analogue-based lineage-tracing in mice showed that β-cell mass adaptation following pregnancy was the result of β-cell replication and that no specialized progenitor cells were involved (81). Partial destruction of the β-cell mass using STZ resulted in an increased β-cell proliferation rate from ~0.6% Ki67 positive β-cells in control to 2.5% in STZ-treated mice 7 days after the treatment (40). In rats, β-cell regeneration following 90% pancreatectomy was associated with an increased mitotic index of β-cells that was about 3 - 4-fold higher than sham animals already after 3 days (82). Several lineage-tracing studies have demonstrated that proliferation of pre-existing β-cells are the major source for β-cell regeneration after a partial (50 – 70%) pancreatectomy or destruction of 70-80% of the β-cell mass in mice (81, 83, 84). The regenerative capacity of β-cell proliferation is

restricted with advanced age. Partial pancreatectomy, STZ and diet-induced obesity did not result in increased β-cell proliferation in aged mice (40, 76).

In humans, it is estimated that in normal individuals the β-cell mass is established in the second or third decade of life (85, 86) and that the highest peak of postnatal β-cell proliferation (1 - 2% of β-cells positive for the proliferation marker Ki67) occurs within the first months of life (22, 87, 88). This means that under normal conditions turnover of adult human β-cells is very low and that individual β-cells are long-lived (22, 85, 86). The reported occurrences of β-cell proliferation in adult human β-cells range from 0 - 0.07% (22, 37, 89, 90). Remarkably, the percentage of replicating β-cells in samples obtained directly at surgery was 0.5% and from frozen biopsies was 0.18% as determined by Ki67 staining (71, 91). This raises the question whether the ability to detect Ki67 is lost during certain conditions of tissue preparation and may therefore explain the differences in β-cell proliferation rates observed.

In humans, the increased β-cell mass observed in obese and pregnant subjects did not correlate with an increase of β-cell proliferation, using Ki67 as a marker for cell proliferation (31, 37, 57). Conversely, a study by Hanley et al. (36) reporting an increased β-cell mass in obese subjects found an increased percentage of β-cells positive for the marker proliferating cell nuclear antigen (PCNA). However, PCNA is also involved in DNA repair, which makes it a less specific marker for cell proliferation than Ki67 (92, 93). These studies have led to the current view that the replicative capacity of adult human β-cells is very limited and that other mechanisms may be responsible for the increased β-cell mass observed in obesity and during pregnancy. However, given the static nature of these cross-sectional studies, it cannot be excluded that the window of β-cell proliferation was missed. Furthermore, an increased number of proliferating β-cells in adult humans have been reported in areas adjacent to gastrinoma (94) and in patients with recent onset type 1 diabetes (95, 96) with a reported β-cell proliferation rate of 0.7% (Ki67 staining) in an 89-years-old patient (96). These studies show that adult human β-cells are able to proliferate under certain conditions.

Altogether the results from human and animal studies show that in both species the rate of β-cell proliferation reduces strongly with advanced age during both basal situations and in response to an increased insulin demand (Table 1). In young rodents, physiological or pathophysiological changes that result in a higher demand for insulin are associated with an increased number of proliferating β-cells. Whether this is also true for β-cell mass adaptation in young humans has not been investigated.

		Baseline	**Obesity/HFD**
Humans	*Young (2 months)*	1 – 2%	Not determined
	Adult (>20 years)	0 – 0.07%	0.02%
Mice	*Young (5 – 6 weeks)*	2.5%	4.5%
	Adult (~1 year)	0.1 – 0.3%	0.1 – 0.3%

Table 1. Reported percentages of β-cell proliferation (detected by Ki67 staining) in mice and humans.

β-Cell apoptosis

Another mechanism by which the β-cell mass can be regulated is apoptosis. In rodent models of diabetes that have a reduced β-cell mass, an increased rate of β-cell apoptosis is observed. In obese mice transgenic for human islet amyloid polypeptide (IAPP) that develop islet amyloid deposits similar to human type 2 diabetes, β-cell proliferation and neogenesis were increased comparable to non-transgenic mice (97). However, in transgenic mice a 10-fold increase in β-cell apoptosis prevented adequate β-cell mass expansion. Also, despite a similar β-cell proliferation in normoglycemic obese Zucker *fa/fa* rats and Zucker diabetic fatty (ZDF) rats, the latter develop diabetes most likely due to an increase in β-cell apoptosis (44). In humans, the decrease in β-cell mass observed in humans with type 2 diabetes is associated with an increased percentage of apoptotic β-cells, about 3-fold in obese and 10-fold in lean diabetic subjects compared to non-diabetic controls (31). Also Yoneda et al. (89) recently reported that the percentage of β-cells positive for the apoptosis marker TUNEL was 0.12% in patients with long-standing type 2 diabetes compared to 0% in healthy controls and newly diagnosed patients.

Reduction of the β-cell mass as a physiological response to a decreased insulin demand is associated with an increase of apoptotic β-cells in rodents. Involution of the β-cell mass in rats that had been infused with glucose for 2 days to expand the β-cell mass, was associated with an increased number of apoptotic β-cells (98). Also, transplantation of insulinomas in rats resulted in a reduction of the endogenous β-cell mass and an increase in β-cell apoptosis (99). Involution of β-cell mass postpartum was associated with an increase in β-cell apoptosis in rats (53). In the pancreas of postpartum women, β-cell apoptosis was rarely detected and similar to non-pregnant women (57). Because of the low frequency of β-cell apoptosis and the small number of women in the post-partum group, no conclusion could be drawn for the involvement of apoptosis in the involution of the β-cell mass in human pregnancy. Altogether, β-cell apoptosis is one of the mechanisms involved in decreasing the β-cell mass in type 2 diabetes. Studies in rodents have shown that β-cell apoptosis also plays a role in normal physiology when involution of the β-cell mass is required.

β-Cell neogenesis

β-Cell neogenesis, or the formation of new β-cells from pancreatic progenitor/stem cells, is a process that occurs during embryonic development of the endocrine pancreas and has been suggested to play a role in normal growth and β-cell adaptation (100). In rodents, regeneration of the endocrine pancreas after pancreatic damage coincides with an increased number of proliferating duct cells that seem to recapitulate embryonic development of the pancreas (101–103). This ductal origin of the regenerating β-cell mass has been challenged by several lineage tracing studies in rodents after pancreatic damage (104–106) and by a recent publication that did not notice regeneration of β-cell mass in response to duct ligation (107). Recently a systematic lineage tracing study showed that β-cell neogenesis predominantly occurs during embryogenesis

and is completely absent in adult mice using different models to stimulate β-cell regeneration: pregnancy, partial pancreatectomy, pancreatic duct ligation and chemical β-cell injury by STZ or Alloxan (108).

Measuring the extent of neogenesis in human cross-sectional histological studies is difficult since there is no marker to identity newly formed cells. The most common criteria for identification of neogenesis are insulin-positive cells in the pancreatic duct epithelium or tiny clusters (1 - 3 cells) of scattered insulin-positive cells in the pancreas (100). During human pancreatic development the percentage of insulin positive cells emerging and/or associated with ductal cells and duct cells positive for insulin is the highest in the prenatal period and drops to ~0.5% postnatal (22, 87). Both human pregnancy and obesity are associated with an increased number of insulin-positive duct cells, ~0.75% in obese and ~1% in pregnant subjects (31, 57). The latter percentage was not reverted postpartum. In patients with impaired glucose tolerance or with newly diagnosed type 2 diabetes an increase in β-cell neogenesis was reported (36, 89) suggesting that this represents an attempt for β-cell mass compensation. Also Mezza et al. (48) recently reported an increased number of cells positive for both insulin and the duct marker CK19 in insulin resistant non-diabetic subjects.

Altogether this has led to the current view that β-cell neogenesis from cells in the ductal compartment occurs during embryogenesis of the endocrine pancreas. However, so far, a major role in β-cell mass regeneration has not been shown using adult animal models (109). The contribution of β-cell neogenesis to β-cell mass adaptation in adult humans remains subject for future investigations.

Transdifferentiation

Transdifferentiation, or direct conversion, is a process characterized by the conversion of one mature cell into another mature cell without an intermediate pluripotent or progenitor state. Previously, it was thought that once cells become fully differentiated they could not switch their phenotype. It was shown that β-cells can be generated by forced expression of key transcription factors from pancreatic non-β-cells (74, 75, 110). The potential of transdifferentiation to contribute to β-cell mass regeneration was shown by Thorel et al. (111) in a model of near-total (>99%) β-cell ablation in which lineage tracing revealed that 65% of the regenerated β-cell mass originated from α-cells. Recently, the β-cell mass of alloxan-induced diabetic mice was regenerated by acinar-to-β-cell reprogramming through transient cytokine exposure (112). These studies have led to the suggestion that transdifferentiation of acinar- or α-cells may contribute to alterations in β-cell mass. In obese non-diabetic human donors, Hanley et al. found an increased number of acinar-associated insulin positive cell clusters that was related to an increased β-cell mass (36). Also, several studies have reported increased numbers of cells positive for both insulin and glucagon in patients with diabetes (113), newly diagnosed diabetes patients (89) or insulin resistant subjects (48). It is evident that there are cells present in the human pancreas

that are positive for both insulin and glucagon. Whether these double positive cells are newly formed endocrine cells derived from progenitor cells or α- or β-cells converting into β- or α-cells, respectively, remains an open question and difficult to assess given the limitations of human tissue samples. Future research should elucidate the role of transdifferentiation in β-cell mass adaptation.

Factors involved in β-cell mass adaptation

Numerous factors have been suggested to play a role in adaptation of the β-cell mass to changes in insulin demand. The first stimulus suggested was obviously glucose, however, the discovery of many hormones and other growth factors that can influence β-cell proliferation and function suggested that glucose has many coworkers (Fig. 4).

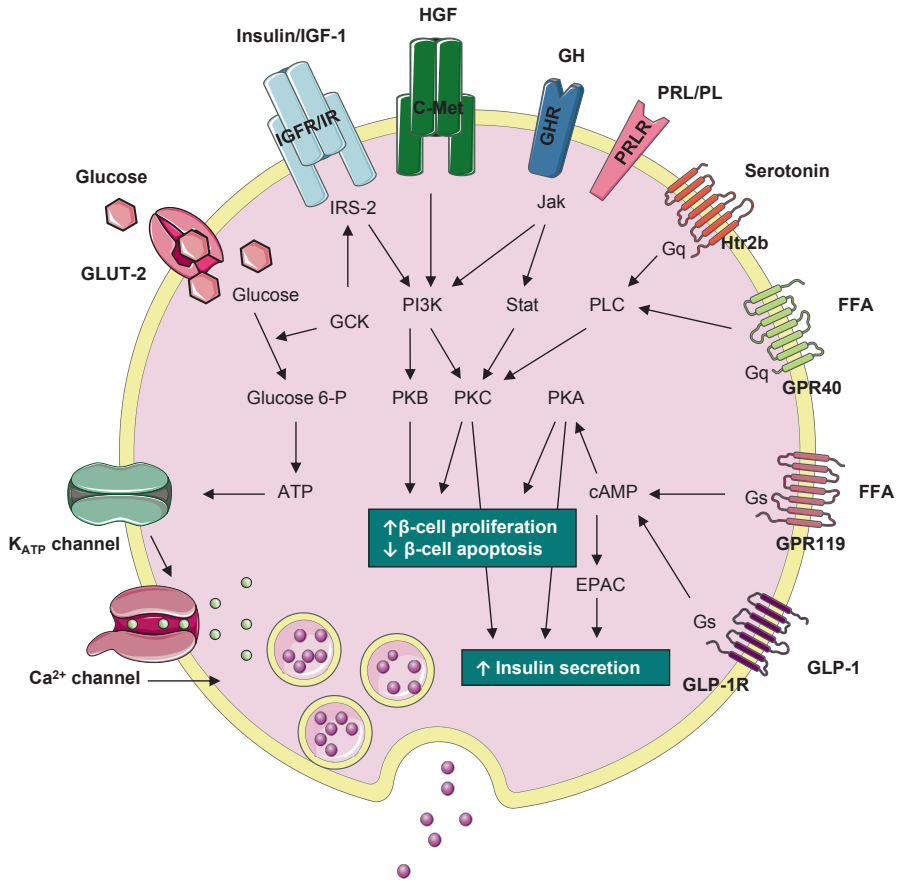

Figure 4. Illustration of several potential factors and some of the multiple signaling pathways that have been reported to be involved in adaptation of β-cells (16, 114, 115). Oxidative metabolism of glucose entering the cell via glucose transporter 2 (Glut-2) leads to an increase in the ATP/ADP ratio. This results in closure of ATP-dependent potassium channels. The subsequent membrane depolarization results in an influx of Ca^{2+} through the opening of voltage-gated calcium-channels. The increase in intracellular calcium concentration stimulates the fusion of insulin granules to the cell membrane and exocytosis of insulin. Increased glucokinase (GCK) activity and activation of the insulin/IGF-1 receptor (IGFR/IR) lead to phosphorylation of insulin receptor substrate 2 (IRS-2) activating a cascade of downstream molecules including phosphoinositide 3-kinase (PI3K) and protein kinase B (PKB) that are associated with increased β-cell proliferation and decreased β-cell apoptosis. Hepatocyte growth factor (HGF) binding to the HGF receptor (HGFR) is also associated with β-cell proliferation via activation of PI3K/PKB signaling. The binding of growth hormone (GH) to the GH receptor (GHR), and prolactin (PRL) or placental lactogen (PL) to the prolactin receptor (PRLR), are associated with activating Jak/Stat signaling pathway leading to activation of protein kinase C (PKC) that is associated with increased β-cell proliferation, decreased β-cell apoptosis, and enhanced insulin secretion. Activation of the G_q-protein coupled receptors Htr2b and GPR40 leads to activation of phospholipase C (PLC), which then activates PKC. Binding of glucagon-like peptide-1 (GLP-1) to the G_s-protein coupled receptor for GLP-1 (GLP-1R) leads to an increase in cAMP levels. cAMP signals are transduced via the exchange protein activated by cAMP (EPAC) or cAMP-dependent protein kinase A (PKA) leading to augmentation of glucose-induced insulin secretion. Activation of PKA also activates signaling pathways involved in β-cell proliferation and survival. Activation of the G_s-protein coupled receptor GPR119 is associated with increased levels of cAMP and improved insulin secretion.

Glucose and insulin

Infusion of glucose results in an increase of the β-cell mass in rats (98, 116). However, there is much debate whether it is glucose itself or the accompanying increase of insulin that is the main trigger for β-cell mass to adapt (117). The critical role for insulin signaling in β-cells became apparent in a model of β-cell specific deletion of the insulin receptor (IR) (βIRKO) in mice (118) that results in glucose intolerance and impairment of high-fat diet-induced β-cell mass adaptation (119). Also, agonism of IR results in signaling through insulin receptor substrate (IRS)-1 and IRS-2. Mice globally deficient in IRS-1 become insulin resistant, but not diabetic because of a compensatory growth in β-cell mass whereas failure of compensation in IRS-2 knock-outs results in diabetes (120). Together this points to an important role for insulin-stimulated IR-IRS-2 signaling in β-cell mass adaptation. However, insulin by itself does not lead to an increase in the β-cell mass. Transplantation of insulinomas in rats results in profound hypoglycemia and a reduction of the endogenous β-cell mass (99, 121). Therefore, it is thought that insulin signaling pathways in β-cells play a more permissive role for β-cell expansion (117). A double knockout of the genes encoding insulin 1 and 2 in mice resulted obviously in fetal growth retardation, diabetes and neonatal lethality, however, these mice exhibited an increased islet mass showing that even in absence of insulin β-cell mass can increase (122).

The importance of glucose metabolism for β-cell mass adaptation was shown in mice haploinsufficient for β-cell glucokinase (GCK+/- mice) (123). In β-cells, GCK catalyzes the rate-limiting step in glucose metabolism and is considered to be the glucose-sensor for regulating glucose-induced insulin secretion (117). GCK+/- mice were unable to increase their β-cell mass when challenged with a high-fat diet, an effect that was mediated by IRS-2 (123). This study illustrates the involvement of glucose metabolism in β-cell mass adaptation that includes cross talk to the insulin signaling pathways. More recently, Porat et al. (124) confirmed the key role for glucose metabolism in regulating β-cell proliferation by showing that β-cell specific knockout of GCK in mice decreases β-cell proliferation and mass and that treatment of mice with a GCK activator resulted in increased β-cell proliferation and mass.

Incretins

The observation that intrajejunal infusion of glucose resulted in a higher insulin secretory response compared to an intravenous glucose injection resulted in the hypothesis that the intestinal wall may be the origin of an insulinogenic mechanism (125). The two most important gut hormones responsible for this effect are glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) (126). GIP is secreted by intestinal K-cells, whereas GLP-1 is secreted by intestinal L-cells in response to carbohydrate or fat intake. Both hormones are rapidly inactivated by the enzyme dipeptidyl-peptidase-IV (DPP-IV) *in vivo* (127). Mice with a double knock-out for the GLP-1 receptor (GLP-1R) and the GIP receptor showed less β-cell adaptation in response to high-fat diet feeding than control mice, which emphasizes the role for these hormones in the

regulation of β-cell mass (128). GLP-1R agonists, more than agonists of the GIP receptor, appear to be involved in β-cell survival and regeneration following pancreatic damage by STZ (129).
In animal models of diabetes GLP-1 or GLP-1R agonist treatment results in increased β-cell proliferation and β-cell mass (130, 131). Also, studies have reported that GLP-1R activation improves regeneration of the β-cell mass following partial pancreatectomy and STZ-induced pancreatic damage in rodents (132–134). However, activation or inactivation of the GLP-1R does not attenuate the endogenous β-cell mass adaptation in insulin resistant *ob/ob* mice (131, 135). Also, it has been shown that aging negatively affects the ability of the β-cell mass to expand in response to incretins in mice (40, 76). Altogether this shows that GLP-1 receptor agonism contributes to β-cell mass regeneration in animal models of diabetes, especially in younger rodents.
In the past decade, numerous GLP-1-based therapies have become available for patients with type 2 diabetes, leading to an improvement of glycemic control (136, 137). There is some evidence that this improved glycemic control may partly be attributed to an improvement of β-cell function (138, 139). *In vitro* it has been shown that incretin therapy has a beneficial effect on survival of isolated human islets by decreasing islet-cell apoptosis (140). In pancreas tissue from donors with type 2 diabetes a 6-fold increase in β-cell mass was observed in patients receiving incretin-based therapies (113). Moreover, the β-cell mass was 3-fold higher compared to non-diabetic controls. No difference in β-cell proliferation was observed. However, these β-cells were probably not functional since the patients still had diabetes. This paper has been criticized because of methodological deficiencies, which may limit interpretation of the results (141). Whether incretins or incretin-based therapies can increase or stabilize the β-mass in adult patients with diabetes remains an open question.

Adipose tissue-derived factors

Obesity is associated with an increased release of free fatty acids (FFA), adipokines such as leptin and resistin, and proinflammatory cytokines from adipose tissue that may affect insulin sensitivity of the muscle and liver leading to insulin resistance (16). Some of these factors have also been described to affect β-cell mass regulation. Leptin is a key hormone in the control of food intake, energy expenditure, metabolism, body weight, and glucose homeostasis. *Ob/ob* and *db/db* mice, which have defects in leptin or in the leptin receptor, respectively, become severely obese, insulin resistant and have an increased β-cell mass. Since β-cells express leptin receptors, leptin has been implicated as a negative regulator of β-cell mass (142). In a mouse model of pancreas-specific (using the Pdx1 promoter) knock-out of the leptin receptor β-cell mass adaptation was hampered after feeding a high-fat diet may point to a direct effect of leptin on β-cell turnover (143). Furthermore, Park et al. (144) reported that central infusion of resistin increased β-cell mass by β-cell proliferation in pancreatectomized diabetic rats. Also, β-cells abundantly express the nutrient sensing G-protein coupled receptor GPR40, which can bind medium- and long-chain

fatty acids leading to glucose-dependent insulin secretion (145, 146). Similarly, the 'fat sensor' GPR119 is highly expressed on β-cells and activation results in release of incretins leading to enhancement of insulin secretion (147, 148). Whether activation of these FFA receptors can also directly affect β-cell mass and proliferation remains to be investigated. Altogether these studies show that several adipose tissue-derived factors can directly modulate β-cell function and mass.

Liver-derived factors

The liver plays a central role in glucose homeostasis, as it is one of the primary sites for storage of glucose and generation of glucose from noncarbohydrate precursors, processes that are regulated by insulin and glucagon (8). Liver-specific insulin receptor knock-out (LIRKO) mice have severe insulin resistance and marked hyperinsulinemia due to an increased β-cell mass (47). This suggests that the liver plays an important role in regulating the β-cell mass. It was shown that regulation of β-cell mass occurred through neuronal signals from the liver (149). Furthermore, several factors produced in the liver have been reported to increase β-cell mass adaptation. Hepatocyte growth factor (HGF) is increased in mice fed a high-fat diet and pharmacological inhibition of HGF resulted in impaired β-cell mass adaptation to diet-induced obesity (150). It was shown that one of the stimuli for β-cell adaptation in LIRKO mice, is a systemic hepatocyte-derived growth factor(s) that was also able to increase proliferation of human β-cells *in vitro* (151). Finally, chemically induced insulin resistance resulted in the discovery of betatrophin, a protein that is enriched in liver and fat tissues, and potently stimulates β-cell proliferation (152). Also, this study reported that expression of increased betatrophin in the liver of pregnant mice and in diabetic *ob/ob* and *db/db* mice, which emphasizes its involvement in β-cell mass adaptation (152). However, transplantation of human islets into insulin resistant mice with elevated concentrations of betatrophin, did not enhance human β-cell proliferation (153). Recently, a study reported that both patients with type 1 and type 2 diabetes have increased circulating concentrations of betatrophin compared to healthy controls (154, 155). This may suggest that a potential stimulus for β-cell proliferation is present in patients with diabetes; however, this is insufficient to increase the number of β-cells.

Pregnancy-related factors

Placental lactogen and prolactin are both members of the growth hormone/prolactin/placental lactogen family and have been described to be involved in the regulation of β-cell mass adaptation during pregnancy in rodents. Placental lactogen is secreted by the placenta and prolactin and growth hormone by the pituitary gland. Placental lactogen and prolactin can both bind the prolactin receptor (PRLR), which is expressed on β-cells (156). In rats, gene expression of PRLR and the growth hormone (GH) receptor were increased in the pancreas during pregnancy (157). Recently it was reported that deletion of the GH receptor in β-cells was associated with a lack of compensatory β-cell mass adaptation in response to HFD-induced obesity (158). β-Cell

proliferation in pregnant mice followed the same temporal pattern as serum concentrations of placental lactogen, suggesting a causal relationship (80). Overexpression of placental lactogen in normal mice resulted in increased β-cell proliferation and islet mass that was associated with hypoglycemia (159). Also, β-cell proliferation and β-cell mass adaptation was impaired in pregnant mice carrying a heterozygous PRLR null mutation (160). Furthermore, it was demonstrated that lactogenic signaling is associated with an increase in serotonin production by β-cells that activates β-cell proliferation in a paracrine/autocrine way (161). Inhibition of serotonin synthesis blocks β-cell mass expansion in pregnant mice. Interestingly, during pregnancy the expression of the stimulatory G_q-coupled serotonin receptor Htr2b was high but normalized at the end of gestation, while expression of the inhibitory G_i-linked serotonin receptor Htr1d was increased shortly before parturition and associated with cessation of β-cell proliferation and regression of β-cell mass (161). This suggests that the effect of serotonin on β-cell adaptation can be modulated by a shift in the receptor expression.

α-Cell mass adaptation

When in 1921 Frederick Banting and Charles Best tested their first crude pancreatic extract in a pancreatectomized dog, which would lead to the Nobel Prize awarded discovery of insulin, they noticed mild hyperglycemia preceding the insulin-induced hypoglycemia. This was attributed to the presence of a substance that mobilized glucose, therefore named 'glucagon' (9, 162). Until recently, most research has focused on how β-cells adapt to physiological and pathophysiological changes in the glucose metabolism. Therefore, little is known about α-cell adaptation during changing metabolic demands. Both type 1 and type 2 diabetes are characterized by a disrupted glucagon-insulin balance due to an absolute or relative hypoinsulinemia leading to insufficient suppression of glucagon secretion (9, 163). The subsequent (relative) hyperglucagonemia aggravates the consequences of hypoinsulinaemia because of an increased glucose output from the liver (9). This is illustrated by the observation that glucagon receptor knockout mice are protected against the development of streptozotocin-induced diabetes in mice (164). Also, GLP-1RA decreases glucagon secretion, which is one of the mechanisms by which this therapy improves glucose homeostasis in patients with type 2 diabetes (165). In non-human primates that spontaneously develop insulin resistance associated with obesity and type 2 diabetes, an increased islet amyloid deposition is associated with increased α-cell proliferation leading to an imbalance in the α- to β-cell ratio (166). Recently this same group showed that in overweight insulin-resistant baboons the α-cell volume was significantly increased, even preceding changes in β-cell volume (167). Also in mice fed a high-fat diet for 8 weeks, α-cell mass was increased in the absence changes in β-cell mass (168). In patients with type 2 diabetes a higher α- to β-cell ratio has been reported, that is due to a decrease in β-cell mass rather than an increase in α-cell

mass (169). In this study the α- to β-cell ratio did not change when comparing obese versus non-obese individuals. This suggests that the extent of α-cell mass adaptation is similar to β-cell mass adaptation.

Aims and structure of this thesis

The aim of the research described in this thesis was to investigate β- and α-cell adaptation in response to different metabolic changes.

Although it has been recognized for a long time that the pancreas is a regionally heterogeneous organ, it is unknown whether β-cell adaptation also occurs heterogeneously throughout the pancreas. **Chapters 2 – 4** describe studies in which we assessed whether β-cell adaptation is topologically homogenous throughout the pancreas in response to an increased demand for insulin in different species. In **chapter 2**, we examined early events of β-cell adaptation in different regions of the pancreas of high-fat diet induced insulin resistant mice. **Chapter 3** describes β-cell adaptation throughout the pancreas in dexamethasone-induced insulin resistant rats. Glucocorticoid-induced insulin resistance occurs within 5 days of treatment (170) and is therefore an acute stimulus for β-cell adaptation. In **chapter 4**, findings from rodent studies are translated to humans. In this chapter we examined β- and α-cell adaptation in different regions of the pancreas from lean and obese human donors.

In **chapter 5**, we studied the effect of one of the most potent stimuli involved in the regulation of β-cell mass and function, GLP-1R activation, on β- and α-cell adaptation under normoglycemic conditions in mice. In animal models of diabetes, incretin-based therapies increase β-cell mass. GLP-1R agonist treatment is also associated with a reduced blood pressure, improved lipid profiles and endothelial function and may therefore also be of benefit for non-diabetic individuals with obesity or cardiovascular disease (171–174). However, the effect of GLP-1R agonist under normoglycemic conditions on β- and α-cells is unclear.

Chapter 6 describes a study in which the influence of a long-term high-fat low-carbohydrate ketogenic diet on glucose tolerance and β- and α-cell adaptation in mice was assessed. Nutrition plays an important role in the development of diabetes and can directly affect β-cell growth and function (98, 115, 116, 145, 147). In many popular diets the amount of fat is substantially increased at the cost of carbohydrates. Thereby the body is forced to use fats instead of carbohydrates as a primary source of energy. We investigated whether these changes in glucose metabolism are associated with changes in β- and α-cells on the long term.

Diabetes mellitus results from an absolute or relative deficiency of functional β-cells leading to an impaired glucose homeostasis. For patients with diabetes, therapies are needed that restore, maintain or prevent loss of functional β-cells. Insight in mechanisms relevant for the protection or improvement of β-cell function is therefore important. Currently, there is no robust technique

available to measure the β-cell mass or function of humans *in vivo*. Also, existing *in vitro* assay platforms are mostly using rodent-derived cell lines and are set up to assess insulin gene expression or protein content (175–178). Following glucose stimulation only a fraction of the total insulin content is secreted from β-cells, which makes these existing read-outs poor indicators of secretory function (14). Therefore, there is a strong need for a robust assay platform using human islets to study β-cell function in order to find novel mechanisms involved in human β-cell function and adaptation to changing metabolic demands. In **chapter 7** we describe three culture platforms using primary human islets in which β-cell function can be assessed. These platforms can be used for high-throughput screening assays to identify novel mechanisms involved in β- and α-cell adaptation.

Chapter 8 summarizes the findings and aims to place various aspects of this thesis in the context of current literature about β- and α-cell adaptation.

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Chapter 2

Topologically heterogeneous β-cell adaptation in response to high-fat diet in mice

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Abstract

Aims

β-Cells adapt to an increased insulin demand by enhancing insulin secretion via increased β-cell function and/or increased β-cell number. While morphological and functional heterogeneity between individual islets exists, it is unknown whether regional differences in β-cell adaptation occur. Therefore we investigated β-cell adaptation throughout the pancreas in a model of high-fat diet (HFD)-induced insulin resistance in mice.

Methods

C57BL/6J mice were fed a HFD to induce insulin resistance, or control diet for 6 weeks. The pancreas was divided in a duodenal (DR), gastric (GR) and splenic (SR) region and taken for either histology or islet isolation. The capacity of untreated islets from the three regions to adapt in an extrapancreatic location was assessed by transplantation under the kidney capsule of streptozotocin-treated mice.

Results

SR islets showed 70% increased β-cell proliferation after HFD, whereas no significant increase was found in DR and GR islets. Furthermore, isolated SR islets showed twofold enhanced glucose-induced insulin secretion after HFD, as compared with DR and GR islets. In contrast, transplantation of islets isolated from the three regions to an extrapancreatic location in diabetic mice led to a similar decrease in hyperglycemia and no difference in β-cell proliferation.

Conclusions

HFD-induced insulin resistance leads to topologically heterogeneous β-cell adaptation and is most prominent in the splenic region of the pancreas. This topological heterogeneity in β-cell adaptation appears to result from extrinsic factors present in the islet microenvironment.

Introduction

The insulin producing pancreatic β-cells are essential to maintain blood glucose levels within a narrow range. When the demand for insulin is chronically increased by physiological or pathological changes, β-cells can adapt by enhancing insulin secretion via increased β-cell function and/or increased β-cell mass (1, 2). Inadequate adaptation leads to the development of hyperglycemia and eventually diabetes mellitus (3, 4). Therefore, insight into the mechanisms that control β-cell adaptation is important for developing therapies that can preserve or enhance β-cell mass.

The pancreas is a regionally heterogeneous organ. During embryonic development the pancreas originates from two epithelial buds. The ventral bud gives rise to the posterior part of the head and the uncinate process, and the dorsal bud forms the anterior part of the head, the body and the tail of the pancreas (5, 6). Pancreatic islets derived from the ventral bud contain more cells producing pancreatic polypeptide (PP), whereas islets derived from the dorsal bud contain more α-cells and secrete more insulin upon glucose stimulation (7, 8). Furthermore, several histological studies in human pancreas describe a higher islet density in the tail compared to the body region of the pancreas (4, 9, 10).

While morphological and functional heterogeneity between individual islets exists, it is unknown whether there are regional differences in β-cell adaptation throughout the pancreas. Regional heterogeneity in cell proliferation rate is observed in regenerating liver lobules after partial hepatectomy (11). In this study, we examine early events of β-cell adaptation in different regions of the pancreas using a model of high-fat diet induced insulin resistance in mice that is known to increase β-cell mass in the long term (12, 13).

Research design and methods

Animals

Male C57BL/6J mice, 8 weeks old (Charles River Laboratories, Wilmington, MA, USA) were fed a high-fat diet (HFD, 45 kcal% fat, D12451, Research Diets, New Brunswick, NJ, USA) or a normal diet (control, 10 kcal% fat, D12450B, Research Diets) for 6 weeks. Average food intake was determined per cage housing 3-4 mice weekly. For the 12-week diet study, 12 week old male C57BL/6J mice (Animal Facility Leiden University Medical Center), that were fed a high-fat or normal diet, were used. For islet transplantation experiments we used male C57BL/6J donor and recipient mice, 8-10 weeks old and fed regular chow. Animal experiments were approved by the ethical committee on animal care and experimentation of the Leiden University Medical Center (Permit Numbers: 09174, 07145, and 11146).

Glucose and insulin tolerance test

An intra-peritoneal glucose tolerance test (GTT) was performed in overnight-fasted mice. Blood samples were drawn from the tail vein before injecting 2 g/kg glucose and after 15, 30, 60 and 120 minutes. An intra-peritoneal insulin tolerance test (ITT) was performed in animals that had been fasted for 6 hours. After measuring basal blood glucose concentration from the tail vein 0.75 U/kg insulin was injected followed by monitoring of the blood glucose concentrations after 15, 30 and 60 minutes. Blood glucose concentrations were measured using a glucose meter (Accu-Chek, Roche, Basel, Switzerland) and insulin concentrations were measured in 5 µl plasma samples by ELISA (Ultra Sensitive Mouse Insulin ELISA kit, Chrystal Chem, Downers Grove, IL, USA).

Pancreas dissection and islet isolation

The pancreas was dissected, weighed and based on their spatial relation to adjacent organs divided into three parts: the duodenal, gastric and splenic region (Fig. S1) (14, 15). The duodenal region was defined as the section of the pancreas attached to the duodenum, the gastric region as the part attached to the pylorus and stomach and the splenic region as the part attached to the spleen. For immunohistochemistry each pancreatic region was fixed in a random orientation in 4% paraformaldehyde and embedded in paraffin. For islet isolation the pancreas of 6-8 mice were pooled per region and digested using 3 mg/ml collagenase (Sigma-Aldrich, St Louis, CA, USA). Islets were manually picked and tested for insulin secretion or purified by gradient separation (1.077 g/ml ficoll, hospital pharmacy, LUMC) and transplanted after overnight culture.

RNA preparation and real-time PCR

Total RNA was extracted from isolated islets using RNeasy micro kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Total RNA (400 ng) was reverse transcribed using M-MLV reverse transcriptase (Invitrogen). Quantitative PCR (qPCR) was performed on a CFX384 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) using the SYBR Green PCR Master Mix (Applied Biosystems, Foster city, CA, USA). Fold induction was calculated using deltaCT method with mouse cyclophilin as housekeeping gene. Mouse primers used were: cyclophilin (forward) 5'-CAGACGCCACTGTCGCTTT-3' and (reverse) 5'-TGTCTTTGGAACTTTGTCTGCAA-3'; cyclin D1 (forward) 5'- TCCGCAAGCATGCACAGA-3' and (reverse) 5'-GGTGGGTTGGAAATGAACTTCA-3'; insulin 2 (forward) 5'-CTGGCCCTGCTCTTCCTCTGG-3' and (reverse) 5'CTGAAGGTCACCTGCTCCCGG-3'.

Glucose-induced insulin secretion

Groups of 10 islets were incubated in a modified Krebs-Ringer Bicarbonate buffer (KRBH) containing 115 mM NaCl, 5 mM KCl, 24 mM $NaHCO_3$, 2.2 mM $CaCl_2$, 1 mM $MgCl_2$, 20 mM HEPES, 2 g/l human serum albumin (Cealb, Sanquin, The Netherlands), pH 7.4. Islets were

successively incubated for 1 hour in KRBH with 2 mM and 20 mM glucose at 37°C. Insulin concentration was determined in the supernatants by ELISA (Mercodia, Uppsala, Sweden). Insulin secretion was corrected for DNA content to correct for islet size differences. DNA content was determined by Quant-iT PicoGreen dsDNA kit (Invitrogen, Carlsbad, CA, USA).

Islet transplantation

For islet transplantation experiments recipient mice were made diabetic by intra-peritoneal injection of 160 mg/kg streptozotocin (STZ, Sigma-Aldrich), freshly dissolved in citrate buffer (pH 4.5). Mice were considered diabetic when the blood glucose concentration was greater than 20 mmol/L. Blood glucose concentrations were determined in blood obtained from the tail vein by a glucose meter (Accu-Chek). Prior to the transplantation mice were given 0.1 mg/kg buprenorfin (Temgesic, Schering-Plough, Kenilworth, NJ) after which they were anaesthetized using isoflurane and kept warm on a heating pad. The left kidney was exposed by a small opening in the flank of the mouse. A small incision was made in the kidney capsule. Using a Hamilton syringe (Hamilton Company, Reno, CA) and polyethylene tubing (PE50, Becton Dickinson, Franklin Lakes, NJ) siliconized with Sigmacote (Sigma-Aldrich), 150 islets per mouse were transplanted under the kidney capsule. The peritoneum and the skin were sutured and the animals were allowed to recover under a warm lamp. Blood glucose concentrations were monitored every other day after transplantation via blood from the tail vein. The islet graft was removed 10 days post-transplantation, fixed by immersion in a 4% paraformaldehyde solution, embedded in paraffin blocks and sliced into 4 µm sections and mounted on slides.

β-Cell mass morphometry and proliferation

For the identification of β-cells, sections were immunostained with guinea-pig anti-insulin IgG (Millipore, Billerica, MA, USA) or rabbit anti-insulin IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 hour followed by HRP- or AP- conjugated secondary antibodies for 1 hour. Sections were developed with 3,3'-diaminobenzidine tetrahydrochloride (DAB) or liquid permanent red (LPR, Dako, Denmark) and counterstained with hematoxylin.

For determining the β-cell mass, 3-4 insulin-DAB stained sections (200 µm apart) per pancreatic region were digitally imaged (Panoramic MIDI, 3DHISTECH, Hungary). β-Cell area and pancreas area stained with hematoxylin were determined using an image analysis program (Stacks 2.1, LUMC), excluding large blood vessels, larger ducts, adipose tissue and lymph nodes. The area of clusters containing ≥ 4 β-cells was individually measured and used to determine the average β-cell cluster area per pancreatic region. Islet density was determined by dividing the number of β-cell clusters by the (regional) area that was analyzed. β-Cell mass was determined by the percentage of β-cell area to pancreas (regional) area multiplied by the pancreas (regional) weight.

Two techniques were used to identify proliferating β-cells. First, incorporation of 5-bromo-2'-deoxyuridine (BrdU, Sigma-Aldrich) in proliferating β-cells was established by administering 50

mg/kg BrdU subcutaneously twice daily during the final 7 days of the 6-week study period. The transplanted recipient mice received 1 mg/ml BrdU in the drinking water (refreshed every other day) during the final 7 days. Sections were double stained for insulin-LPR and BrdU (BrdU staining kit, Invitrogen). BrdU-positive β-cells were assessed as a proportion of all β-cells per pancreatic region or islet graft. Second, sections were double stained with goat anti-Ki67 IgG (Santa Cruz Biotechnology) and guinea-pig anti-insulin IgG (Millipore) overnight at 4ºC after heat-induced antigen retrieval in 0.01 M citrate buffer (pH 6.0) followed by biotin-conjugated anti-goat IgG (Dako), streptavidine-Alexa 488 (Invitrogen) and TRITC-conjugated anti-guinea-pig (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Nuclei were stained with DAPI (Vector Laboratories, Burlingame, CA, USA). Apoptotic beta-cells were counted after being identified by immunostaining for insulin and by the terminal deoxynucleotidyl-transferase-mediated deoxyuridine 5-triphosphate nick end labeling (TUNEL) assay (Roche). The investigator was blind to the experimental conditions.

Statistical analysis

Data are presented as means ± SEM. Statistical calculations were carried out using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). The statistical significance of differences was determined by an unpaired Student's *t* test or two-way ANOVA, followed by Bonferroni's multiple comparisons test, as appropriate. $P<0.05$ was considered statistically significant.

Results

Metabolic characteristics of mice fed HFD for 6 weeks

Body weight and food intake were increased after 6 weeks HFD compared to control (Fig. 1A, B). After overnight fasting glucose concentrations were similar (5.1 ± 0.2 mmol/L (HFD) vs 5.0 ± 0.2 mmol/L (control), $p=0.72$). Glucose tolerance was decreased in HFD mice compared to control (Fig. 1C, D), whereas insulin concentrations were increased twofold (Fig. 1E, F). Insulin tolerance was decreased by HFD (Fig. 1G, H). Therefore, HFD for 6 weeks is sufficient to induce insulin resistance leading to an increased demand for insulin from β-cells.

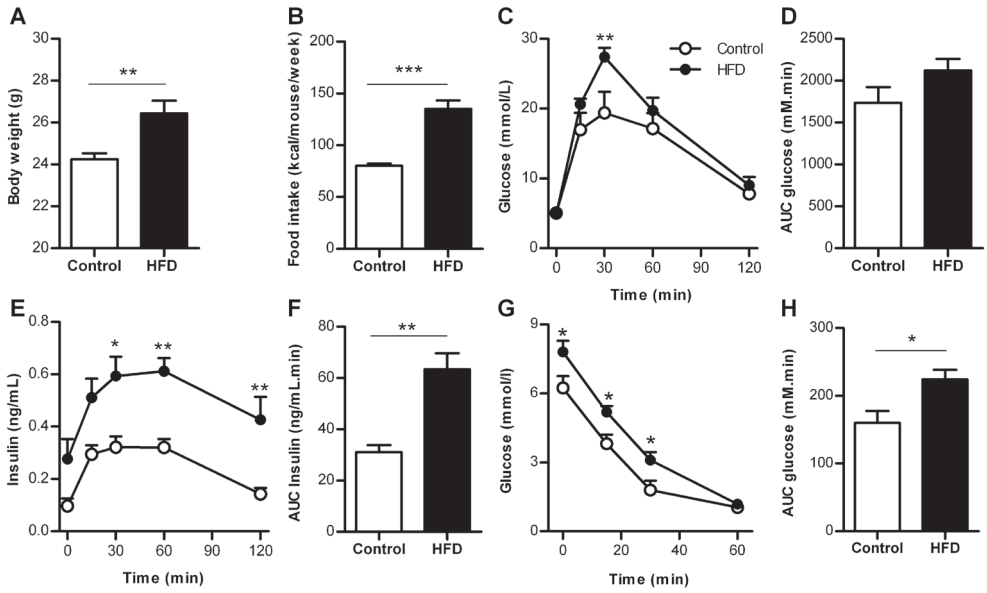

Figure 1. Metabolic characteristics of control mice and mice fed a high fat diet for 6 weeks. A. Body weight (n = 13-14 mice). B. Food intake (n = 4 cages). C. Blood glucose concentrations during GTT (n = 6 mice). D. AUC blood glucose concentrations during GTT (n = 6 mice). E. Insulin concentrations during GTT (n = 5-6 mice). F. AUC insulin concentrations during GTT (n = 5-6 mice). G. Blood glucose concentrations during ITT (n = 8 mice). H. AUC of glucose concentrations during ITT (n = 8 mice). HFD = high-fat diet, AUC = area under the curve. *p<0.05, **p<0.01, ***p<0.001.

Increased β-cell proliferation in the splenic region of the pancreas in response to HFD

The effect of HFD on early β-cell adaptation in different regions was evaluated. The pancreas was divided in three regions: a duodenal, gastric and splenic region. The β-cell mass was determined by analyzing 29.9 ± 1.6 mm^2 pancreatic tissue per region per mouse. A difference in β-cell mass between HFD and control mice was found neither in the entire pancreas, nor in the separate regions (Fig. 2A, B). For determination of the average β-cell cluster area 46 ± 2.5 clusters were included per region. The β-cell cluster area was significantly larger in islets from the GR, no differences were found between HFD and control mice (Fig. 2C, D). Islet density was homogeneous throughout the pancreas and similar after HFD for 6 weeks (Fig. 2E, F). After 12 weeks HFD we could confirm an increased beta-cell area, which was mostly augmented in the SR of the pancreas compared to control mice (Fig. 2G, H).

For determination of the number of proliferating β-cells, Ki67+ β-cells were counted in 95 ± 6 islets per mouse (Fig. 3A). The occurrence of Ki67+/insulin+ cells was very low (Ki67+/Insulin+ 0.12 ± 0.03 % (HFD) vs. 0.09 ± 0.02 % (control)). To increase the sensitivity for detecting proliferating β-cells, BrdU was administered for 7 days. We counted 885 ± 48 β-cells per region per mouse. β-Cell proliferation was significantly increased in HFD mice compared to control mice (Fig. 3C). A positive correlation was found between the increase in body weight and the rate of β-cell

proliferation in HFD mice (r^2=0.70; p<0.05) (data not shown). HFD increased β-cell proliferation by 70% in the SR of the pancreas whereas no significant increase was found in the DR and GR (Fig. 3B, D). Also mRNA levels of Cyclin D1 were increased in islets from the splenic region of HFD mice (Fig. 3E). By counting on average 460 ± 73 cells per region per mouse, the occurrence of apoptotic β-cells was very low and not different between the two groups (data not shown).

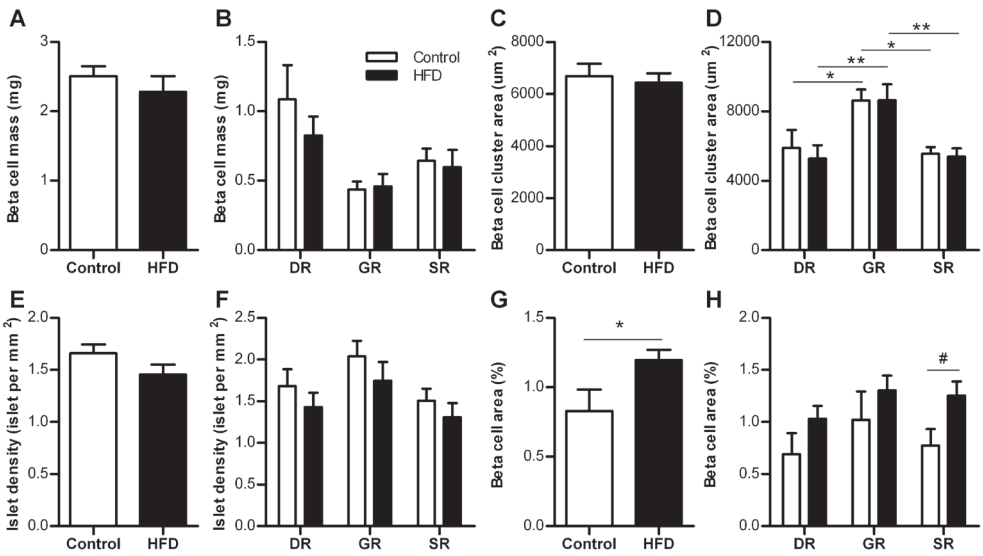

Figure 2. β-Cell mass morphometry in control and HFD mice. A. β-Cell mass in the entire pancreas after 6 weeks (n = 6 mice). B. β-Cell mass by pancreatic region after 6 weeks (n = 6 mice per region). C. β-Cell cluster area in the entire pancreas after 6 weeks (n = 6 mice). D. β-Cell cluster area by pancreatic region after 6 weeks (n = 6 mice per region). E. Islet density in the entire pancreas after 6 weeks (n = 6 mice). F. Islet density by pancreatic region after 6 weeks (n = 6 mice per region). G. β-Cell area in the entire pancreas after 12 weeks (n = 6 mice). H. β-Cell area by pancreatic region after 12 weeks (n = 6 mice per region). DR = duodenal region, GR = gastric region, SR = splenic region, HFD = high-fat diet. *p<0.05, $^{\#}p$<0.05 by unpaired Student's t test.

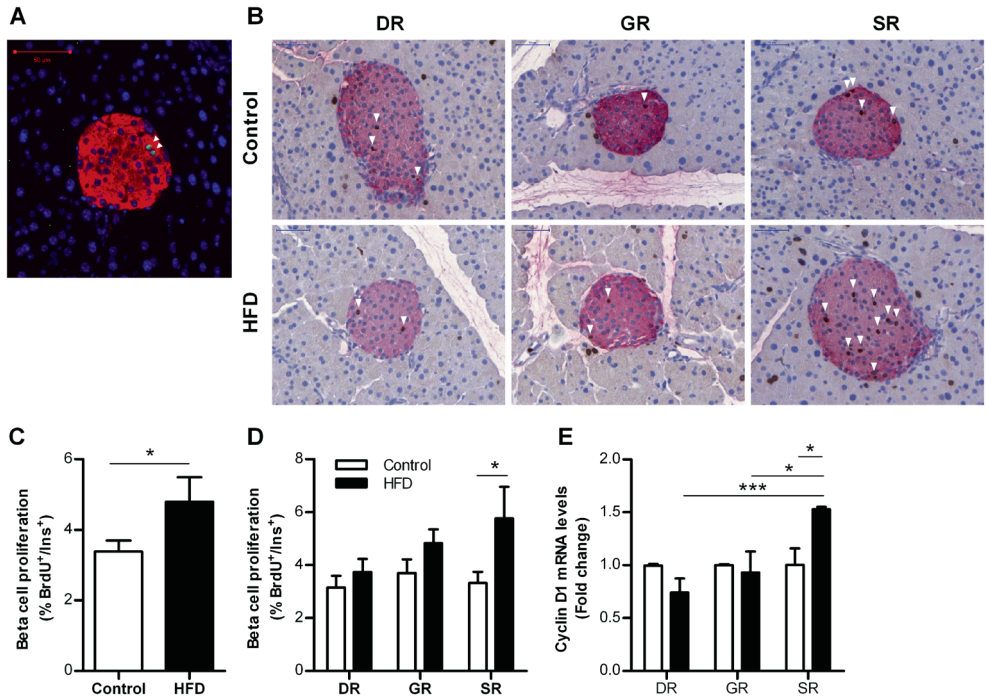

Figure 3. β-Cell proliferation in control and HFD mice after 6 weeks. A. Image of proliferating beta-cells (arrowheads), Ki67 (green), insulin (red) and DAPI (blue). Scale bar = 50 µm. B. Image of proliferating beta-cells (arrowheads), BrdU (brown) and insulin (red) per pancreatic region in control and HFD mice. Mice received BrdU during the final 7 days. Scale bar = 50 µm. C. β-Cell proliferation in the entire pancreas, BrdU labeling during the final 7 days (n = 6 mice). D. β-Cell proliferation by pancreatic region, BrdU labeling during the final 7 days (n = 6 mice per region). E. Cyclin D1 mRNA expression by pancreatic region, control = 1. DR = duodenal region, GR = gastric region, SR = splenic region, HFD = high-fat diet. *p<0.05, ***p<0.001

Prominent increase in glucose-induced insulin release from isolated islets in the splenic region by HFD

The functional adaptation of islets from HFD mice was assessed by measurement of glucose-induced insulin secretion. Stimulation of islets from HFD mice with 20 mM glucose led to a twofold increase in insulin secretion compared to control mice (Fig. 4A). When comparing the response of islets derived from the different pancreatic regions after HFD, insulin secretion was 56% and 72% higher in SR islets compared to GR and DR islets, respectively (Fig. 4B, C). Expression levels of Insulin mRNA showed a similar pattern (Fig. 4D).

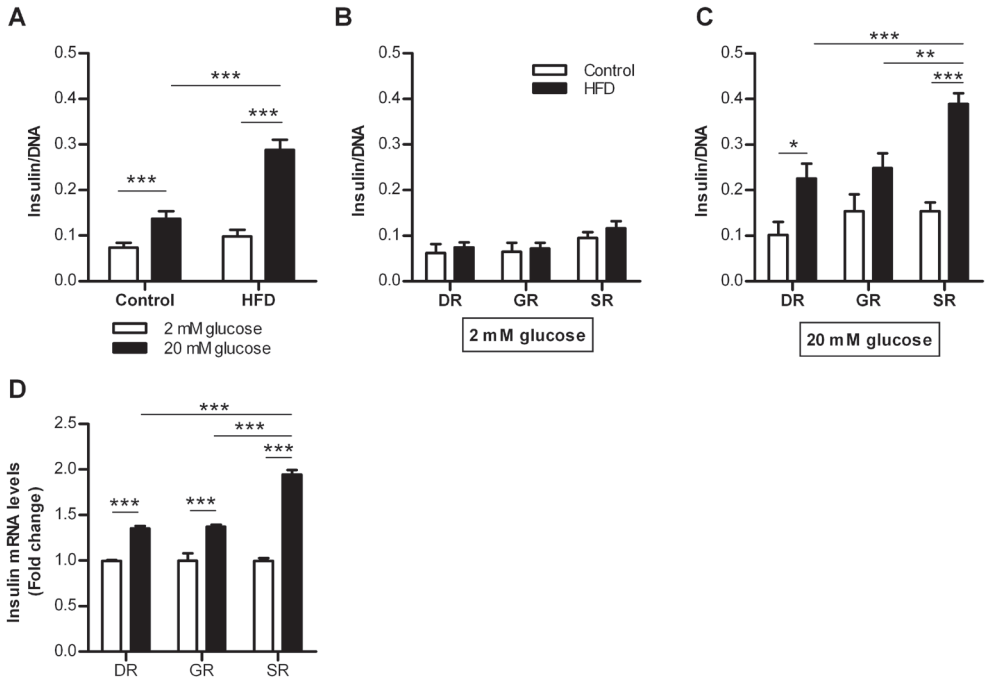

Figure 4. Glucose-induced insulin secretion from isolated islets of control and HFD mice. Insulin secretion was corrected for DNA content. A. Insulin secretion during 2 mM and 20 mM glucose stimulation from islets in the entire pancreas (n = 24). B. Insulin secretion from islets by pancreatic region during incubation in 2 mM glucose buffer (n = 8 per region) and C. 20 mM glucose buffer (n = 8 per region) for control and HFD mice. D. Insulin mRNA expression by pancreatic region, control = 1. DR = duodenal region, GR = gastric region, SR = splenic region, HFD = high-fat diet. *p<0.05, **p<0.01, ***p<0.001.

Similar islet function and β-cell proliferation after transplantation of islets from different regions to an extrapancreatic location

Next we assessed whether the observed proliferative and functional islet heterogeneity is due to differences in the islet microenvironment or due to intrinsic differences between islets from the three regions. Islets isolated from the three pancreatic regions were transplanted under the kidney capsule of syngeneic STZ-induced diabetic mice. All grafts contained 150 handpicked islets with an average size of that was similar for all transplants resulting in a similar graft size (Fig. 5A). Since hyperglycemia in these mice is required for detectable adaptation of grafted islets (16, 17), the increased demand for insulin in this model is expected to be a potent stimulus for β-cell adaptation in the islet graft. The islet graft size was sufficient to reduce blood glucose concentrations, but it was not sufficient to lead to normoglycemia in most mice thereby maintaining the stimulus for β-cells to adapt (17). Normoglycemia (defined as blood glucose concentration <10 mmol/L) was reached with 3 out of 7 DR, 1 out of 6 GR and 2 out of 8 SR islet grafts. On average, all transplants led to a similar decrease in hyperglycemia (blood glucose concentrations 10 days post-transplantation 11.5 ± 1.6 mmol/L (DR grafts), 15.5 ± 2.1 mmol/L (GR grafts), 15.7 ± 1.7

(SR grafts) mmol/L, p=0.20; Fig. 5B, C). For determination of the number of proliferating β-cells in the grafts 1114 ± 113 β-cells per islet graft were counted. β-Cell proliferation was similar for DR, GR or SR islet grafts (p=0.53, Fig. 5D, E).

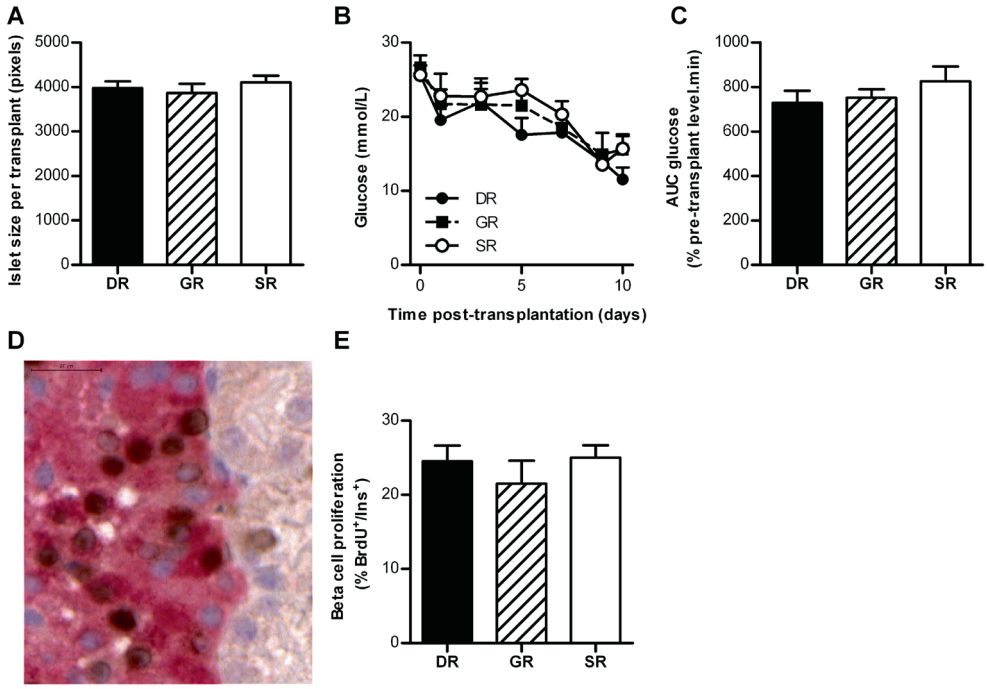

Figure 5. β-Cell adaptation in islets grafts from different pancreatic regions transplanted in syngeneic diabetic mice. A. Average islet size per transplant. B. Blood glucose concentrations of STZ-induced diabetic mice followed up to 10 days after transplantation (n = 6-8 mice per region) of DR, GR or SR islets. C. AUC blood glucose concentrations post-transplantation corrected for pre-transplantation glucose concentration (n = 6-8 mice per region). D. Image of proliferating beta-cells, positive for both BrdU (brown) and insulin (red) in islets transplanted under the kidney capsule of diabetic mice. Scale bar = 20 μm. E. β-Cell proliferation in the islet grafts 10 days after transplantation, BrdU labeling during the final 7 days (n = 6-7 mice per region). DR = duodenal region, GR = gastric region, SR = splenic region, AUC = area under the curve.

Discussion

The main results of our study show that β-cell adaptation is topologically heterogeneous throughout the pancreas. Splenic islets are involved in the first line of response in β-cell adaptation. Although morphological and functional heterogeneity between individual islets have been described before, this is the first study showing regional differences in β-cell adaptation to an increased metabolic demand.

β-Cell adaptation in different pancreatic regions was studied in mice fed HFD for 6 weeks. We hypothesized that this time period would be long enough to induce metabolic changes and would allow us to investigate early islet adaptation. Six weeks HFD led to insulin resistance, with a higher demand for insulin to which β-cells started to adapt. Since β-cell mass was not significantly changed yet, but an increased rate of β-cell proliferation was already observed, this is an appropriate model for studying early events of β-cell adaptation.

The presence of increased β-cell proliferation and an augmented insulin secretory response in islets derived from the splenic region of the pancreas indicates that islets in this part of the pancreas constitute an early line of defense against an increased insulin demand. Our study was not designed to answer whether the relative contribution of β-cell adaptation in the different regions changes during prolonged high-fat feeding.

The observed proliferative and functional heterogeneity between islets from different regions in response to a HFD stimulus could be explained in two ways: either the islets from different pancreatic regions are intrinsically different or they receive distinct extrinsic signals from their microenvironment. This latter hypothesis was investigated by transplanting isolated islets to an extrapancreatic location in diabetic mice. After 10 days, islet grafts from the duodenal, gastric or splenic region led to a similar decrease in hyperglycemia and there was no difference in β-cell proliferation. Therefore we suggest that this newly identified topological heterogeneity of β-cell adaptation observed in HFD mice is most likely the result of distinct extrinsic signals present in the microenvironment of the islet.

The islet microenvironment is formed by a complex network of nerves and blood vessels that mediate neuronal, humoral and circulatory signals which are involved in β-cell adaptation. Islets are densely innervated by the autonomic nervous system (18) and it was reported that β-cell mass adaptation is regulated by neuronal signals from the liver (19). A recent study identified a subpopulation (5%) of islets with greater blood perfusion and vascular density, which was associated with increased β-cell function and proliferation (20). But it still remains unclear whether the increased vascular density is the cause or the consequence of the increased β-cell mass. Another possible factor is the strong paracrine dialogue between the islet microvasculature and β-cells (21). However, here we show that early adaptation persists *in vitro* after isolation of the islets, since SR islets from HFD mice display an enhanced glucose-induced insulin secretion compared to DR and GR islets. Therefore, this indicates that heterogeneity in β-cell adaptation is not dependent on immediate innervation or vascular blood supply.

Furthermore, a strong structural and functional relationship between islets and acinar cells exists, which is referred to as the islet-acinar axis, in which insulin and somatostatin play an important role in regulating exocrine function (22). It was shown that the amylase content of acinar tissue from the splenic region of rats is higher than in the duodenal region of the pancreas (23). Whether the exocrine tissue surrounding islets can locally influence β-cell adaptation remains an open question.

Past studies have shown that islets from the dorsal pancreas secrete more insulin compared to islets from the ventral region (8, 24). The gastric and splenic regions originate from the dorsal lobe. However our data also show heterogeneous adaptation within the dorsal pancreas (GR vs SR) indicating that embryonic origin of the different regions does not entirely explain the heterogeneity observed in this study.

It is likely that our findings of regional heterogeneity in β-cell adaptation can be extended to the human pancreas. In the pancreas of humans and non-human primates heterogeneity in islet density throughout the pancreas (4, 9, 10), and changes in islet mass associated with different metabolic conditions have been described (3, 25–29). For patients undergoing distal pancreatectomy, this would imply loss of the most adaptive islets which may lead to a higher risk for postoperative diabetes. Furthermore, histological studies of β-cell adaptation in the human pancreas are often based on tissue samples from the splenic region only (3, 25), whereas this may not be representative for the entire organ.

Finally, the findings of this study imply that the islet microenvironment harbors factors that are involved in β-cell adaptation. Investigation of these regional differences may lead to the identification of factors that play a key role in β-cell regeneration.

Acknowledgements

The authors thank Jacques Duijs for technical assistance and Maarten Ellenbroek for the illustration shown in Fig. S1.

Supporting Information

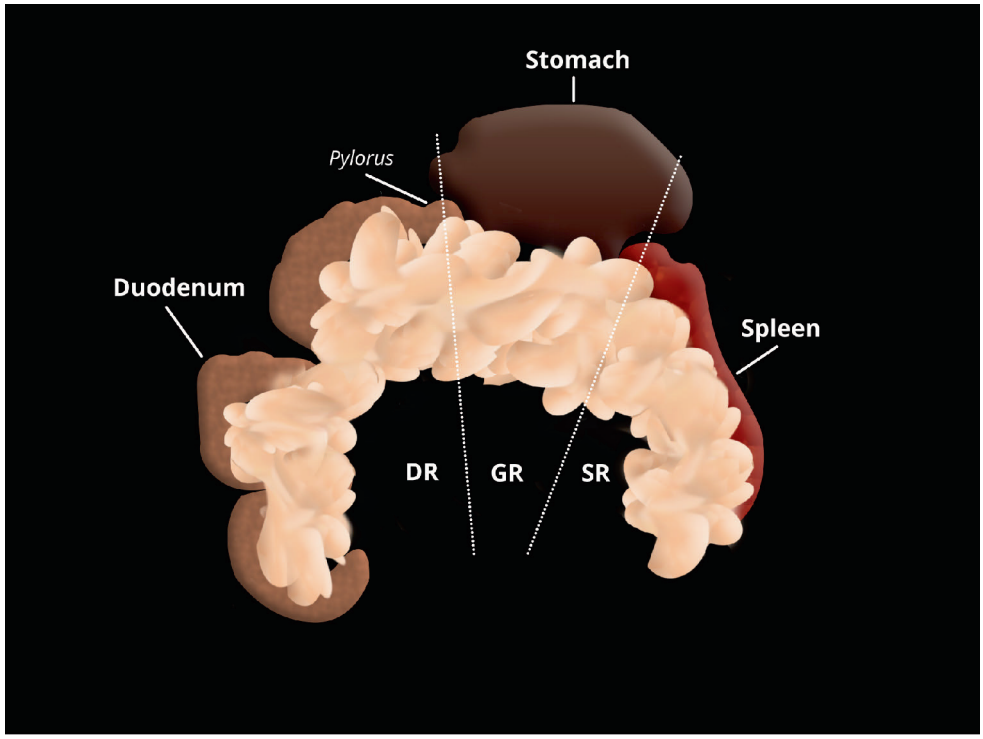

Figure S1. The spatial relation to adjacent organs was used to divide the pancreas into three parts: DR = duodenal region, GR = gastric region, SR = splenic region.

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Chapter 3

β-Cell adaptation in response to dexamethasone-induced insulin resistance is topologically heterogeneous in rats

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In preparation

Abstract

Introduction

β-cells adapt to an increased insulin demand by increasing β-cell function and/or the number of β-cells. Diet-induced insulin resistance in mice leads to topologically heterogeneous β-cell adaptation. It is unknown whether this also occurs in other models of insulin resistance. In this study we investigate β-cell adaptation throughout the pancreas in glucocorticoid-induced insulin resistance in rats.

Methods

Wistar rats were treated with 10 μg/day dexamethasone (DXM) for 3 or 6 weeks. Glucose tolerance was assessed by an intravenous glucose tolerance test (GTT). The pancreas was divided in a duodenal (DR), gastric (GR), and splenic region (SR) and taken for histology. Immunostainings for insulin and Ki67 were performed to identify β-cells and proliferating β-cells, respectively.

Results

After 2 weeks of DXM-treatment the insulin secretory response during the GTT was two-fold increased compared to controls. β-Cell area was significantly increased after DXM-treatment, and this increase was most prominent in the SR of the pancreas. The average β-cell cluster size in the SR of DXM-treated rats was increased, whereas β-cell proliferation was not significantly different.

Conclusion

DXM-induced insulin resistance in rats leads to topologically heterogeneous β-cell adaptation. The splenic region of the pancreas is particularly responsive to changes in insulin resistance in rodents. Comparison of regional differences may lead to the identification of mechanisms involved in β-cell adaptation.

Introduction

The insulin producing β-cells are essential for keeping the blood glucose levels within a narrow range. When insulin sensitivity is chronically reduced by physiological or pathological changes, β-cells can meet the higher demand for insulin by enhancing β-cell function and/or increasing the number of β-cells. Obese non-diabetic subjects have a higher β-cell mass compared to lean subjects (1–3). Also, insulin resistance in animal models of obesity is correlated to a higher β-cell mass (4–6). An inadequate number of functional β-cells contributes to the development of type 2 diabetes (1, 7). Therefore, it is important to elucidate mechanisms involved in β-cell mass adaptation for developing therapies that can preserve β-cell mass.

Glucocorticoids are widely used as therapeutic agents, especially for their anti-inflammatory actions. However, they antagonize the action of insulin and thereby induce insulin resistance (8). In rats and non-human primates glucocorticoid treatment is associated with increased insulin secretion and β-cell mass adaptation (9–13). Glucocorticoid-induced insulin resistance occurs within 5 days of treatment (11) and is therefore an acute stimulus for β-cell adaptation.

The pancreas is a heterogeneous organ. We have recently shown that high-fat diet induced insulin resistance leads to topologically heterogeneous β-cell adaptation in mice (14). β-Cell adaptation was most prominent in the splenic region of the pancreas, suggesting that these islets are the first to respond to changes in the demand for insulin. It is unknown whether this also occurs in other models of insulin resistance. In this study we investigated beta-cell adaptation throughout the pancreas of glucocorticoid-induced insulin resistance in rats.

Materials and Methods

Animals

Experiments were performed in adult male Wistar rats (220-310 g, Harlan, Zeist, The Netherlands) with approval of the Animal Care Committee of the Royal Netherlands Academy of Arts and Sciences. The rats had access to standard diet and water *ad libitum*. All experiments were performed in the rats' home-cage. Rats were treated with 10 µg dexamethasone 21-phosphate disodium salt (Sigma-Aldrich, St Louis, CA, USA) per day in the drinking water for 3 or 6 weeks. Control rats received untreated drinking water for 3 weeks. According to the method of Steffens et al.(15), an intra-atrial silicone catheter was surgically implanted into the left jugular vein of rats that were anesthetized using a mixture of fentanyl/fluanisone (Hypnorm; 1 ml/kg i.m.) and midazolam (Dormicum; 0.3 ml/kg s.c.) 2 weeks before the GTT. After the surgery the animals were placed into an incubator (30°C) until awakening; saline was injected subcutaneously to prevent dehydration.

Glucose tolerance test

An intravenous GTT was performed in 2-hours fasted rats 1 week before sacrifice. A blood sample was drawn (t=0), immediately followed by the infusion of a glucose bolus (25%, 1.0 g/kg BW) into the jugular vein catheter. Subsequently blood samples were collected at t=5, 10, 20, 30 and 60 min after the infusion of the glucose bolus. Plasma glucose concentrations were determined using a glucose/glucose oxidase-Perid method (Boehringer Mannheim, GmGH, Germany). Plasma immunoreactive insulin concentrations were determined using a radio immunoassay kit (Linco Research, St Charles, MO, USA). Area under the curve (AUC) for insulin and glucose were measured for each curve relative to a y-axis value of 0.

Pancreas dissection

The pancreas was dissected, weighed and based on their spatial relation to adjacent organs divided into three parts: the duodenal, gastric and splenic region, as described before (14). The duodenal region (DR) was defined as the section of the pancreas attached to the duodenum, the gastric region (GR) as the part attached to the pylorus and stomach and the part attached to the pancreas was taken as the splenic region (SR). Pancreas tissue was fixed by immersion in a 4% paraformaldehyde solution, embedded in paraffin blocks, sliced into 4 µm sections and mounted on slides. Each pancreatic region was separately embedded, immunostained and analyzed. The average of the three regions was taken as a measure for the entire organ.

β-Cell mass morphology and proliferation

For identification of β-cells, sections were immunostained with rabbit anti-insulin IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 hour followed by anti-rabbit IgG-HRP (DAKO, Glostrup, Denmark). Sections were developed with 3,3'-diaminobenzidine tetrahydrochloride (DAB) and counterstained with hematoxylin. Stained sections were digitally imaged (Panoramic MIDI; (3DHISTECH, Budapest, Hungary). β-Cell area and pancreas area were determined using an image analysis program (Stacks 2.1; LUMC), excluding large blood vessels, larger ducts, adipose tissue, and lymph nodes as previously described (14). β-Cell mass was determined by the ratio of β-cell area to pancreas area multiplied by the pancreas weight. β-Cell cluster size was determined as the average size of β-cell clusters (defined as ≥4 β-cells per cluster) per rat.

To identify proliferating β-cells sections were double stained with mouse-anti Ki67 (Becton Dickinson, Franklin Lakes, NL, USA) and guinea pig anti-insulin (Millipore, Billerica, MA, USA) overnight, after heat-induced antigen retrieval in 0.01 M citrate buffer. Sections were incubated with secondary antibodies biotin anti-mouse (Jackson Immunoresearch Laboratories, West Grove, PA, USA), SA-alexa 488 (Invitrogen, Carlsbad, CA, USA) and TRITC anti-guinea pig (Jackson Immunoresearch Laboratories) for 1 hour. DAPI (Vectashield; Vector Laboratories) was used to visualize the nuclei. Randomly selected islets were digitally imaged using a 20x objective on a fluorescence microscope (Leica Microsystems, Wetzlar, Germany).

Statistics

All data are presented as means ± SE. Statistical calculations were carried out using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). The statistical significance of differences was determined by an unpaired Student's *t*-test or ANOVA, followed by Bonferroni's multiple-comparisons test, as appropriate. $P < 0.05$ were considered statistically significant.

Results

Metabolic characteristics of control and DXM-treated rats

Food- and water intake by the rats were not affected by DXM treatment (data not shown) and body weight between DXM-treated and control rats was similar at time of sacrifice (Table 1). Two weeks of DXM treatment increased the peak of glucose concentration at 5 min (Fig 1A), but the AUC of glucose was not significantly different between the groups (Fig 1B). In contrast, glucose levels during the GTT were normal in 5 weeks DXM treated rats. In both DXM-treated groups the insulin secretory response during the GTT was significantly increased (Fig. 1C and D). DXM treatment led to increased 2-hour fasted plasma insulin levels after 3 weeks and 6 weeks, whereas blood glucose levels were unchanged (Table 1).

	Control	DXM 3 wks	DXM 6 wks	ANOVA
Body weight (g)	354.5 ± 3.0	357.2 ± 10.6	340.9 ± 5.4	p=0.11
Blood glucose (mmol/l)	6.49 ± 0.22	6.10 ± 0.11	6.07 ± 0.13	p=0.17
Plasma insulin (ng/ml)	1.34 ± 0.18	4.17 ± 0.72*	5.18 ± 0.58**	p<0.0001

Table 1. Body weight, blood glucose and plasma insulin levels at time of sacrifice. *p<0.01, **p<0.001 vs. control. DXM = dexamethasone.

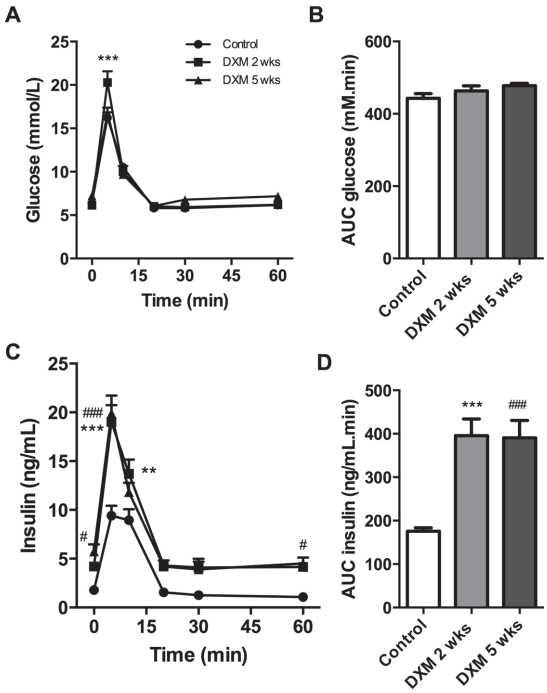

Figure 1. Glucose tolerance in control and 2 or 5 weeks DXM-treated rats. A. Blood glucose concentrations during the glucose tolerance test (GTT) (n = 5-9 rats). B. Area under the curve (AUC) of glucose concentrations during the GTT. C. Insulin concentrations during the GTT (n = 5-9 rats). C. AUC of insulin concentrations during the GTT. DXM = dexamethasone. $^{**}p<0.01$ or *** $p<0.001$ for DXM 2 weeks vs. control; $^{\#}p<0.05$ or $^{\#\#\#}p<0.001$ for DXM 5 weeks vs. control.

DXM-treatment increases β-cell mass

The effect of DXM treatment on β-cell mass was evaluated. Pancreas weight was increased in DXM-treated rats after 3 weeks (control 1.13±0.04 g vs. DXM 3 weeks 1.31±0.06 g, $p<0.05$). The β-cell area was determined by analyzing 23.5±1.1 mm^2 of pancreatic tissue per region per rat. DXM treatment led to a significant increase of the β-cell area after 6 weeks (Fig 2A-C). Also, the β-cell mass was significantly increased in rats treated with DXM for 3 weeks (control 7.9±0.9 mg vs. DXM 3 weeks 12.7±1.5 mg, $p<0.05$). This increase was associated with an increase of the average β-cell cluster size after 6 weeks of treatment (Fig 2D). No difference in islet density was measured (Fig 2E).

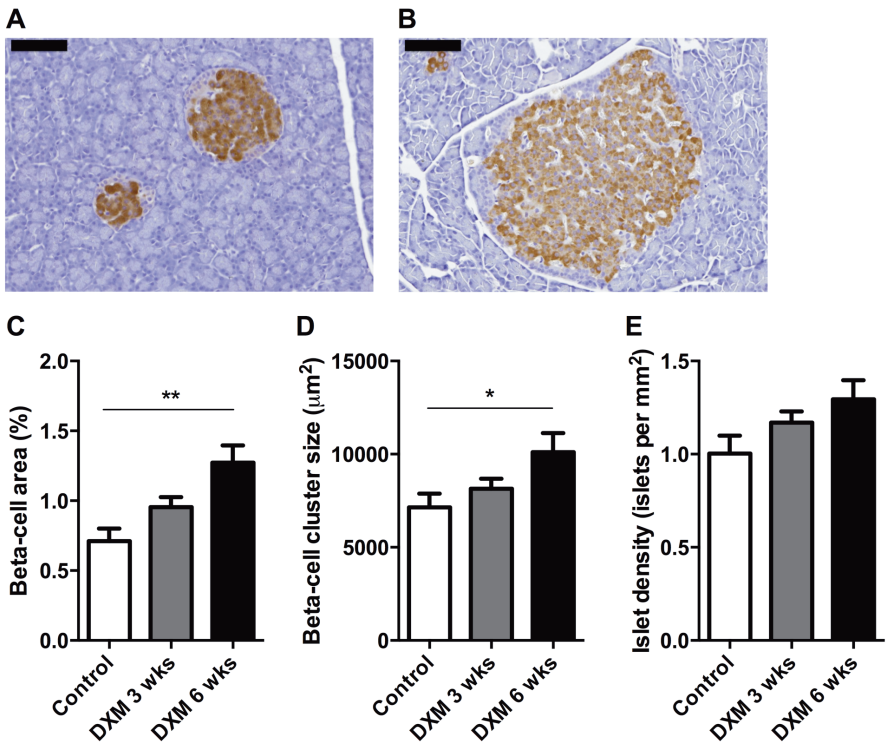

Figure 2. β-Cell mass morphometry in control and DXM-treated rats. A. Representative picture of β-cells (brown) in a control rat. Scale bar = 100 µm. B. Representative picture of β-cells (brown) in a 6 weeks DXM-treated rat. Scale bar = 100 µm C. β-Cell area (n = 5-9). D. Mean β-cell cluster area (n = 5-9). E. Islet density (n = 5-9). DXM = dexamethasone. *p<0.05 by unpaired Student's t test, **p<0.01.

Increased β-cell area in the splenic region of the pancreas in response to DXM treatment

To assess β-cell adaptation throughout the pancreas, the β-cell area, β-cell cluster size and islet density were determined by pancreatic region (i.e. DR, GR and SR). After 6 weeks DXM treatment, the β-cell area in the SR of the pancreas was significantly increased compared to control rats (Fig 3A). This was associated with an increased average β-cell cluster size in the SR of DXM rats treated for 6 weeks (Fig 3B). In contrast, no differences in β-cell area or β-cell cluster size were observed in the DR and GR of the pancreas after DXM treatment (Fig 3A,B). Islet density was similar between DXM-treated and control rats (Fig 3C). The mean area of individual β-cells was unchanged after DXM-treatment in the SR (individual β-cell size 205.1±7.1 µm^2 (control) vs. 189.3±9.1 µm^2 (DXM 3 weeks) vs. 209.1±9.5 µm^2 (DXM 6 weeks), p=0.32). For determination of the proliferating β-cells, we counted the number of Ki67 positive cells out of 928±128 β-cells per region per rat. The frequency of proliferating β-cells was similar between control and DXM-treated rats at 3 and 6 weeks (Fig 3D).

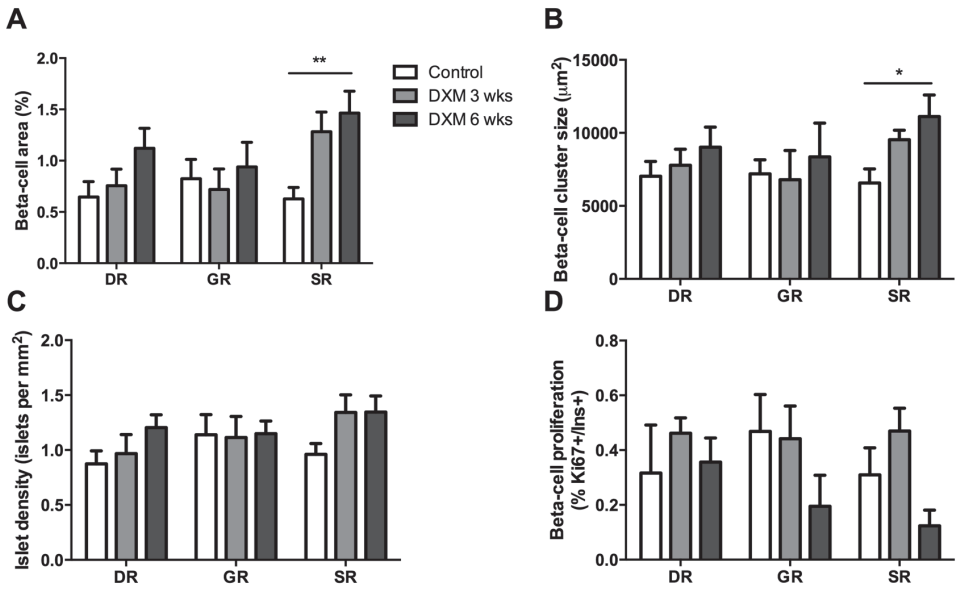

Figure 3. β-Cell morphometry and β-cell proliferation in control and DXM-treated rats by pancreatic region. A. β-Cell area by pancreatic region (n = 5-9). B. β-Cell cluster size by pancreatic region (n = 5-9). C. Islet density by pancreatic region (n = 5-9). D. β-Cell proliferation by pancreatic region (n = 5-8). DXM = dexamethasone, DR = duodenal region, GR = gastric region, SR = splenic region. *p<0.05 by one-way ANOVA, **p<0.01.

Discussion

The main finding of our study is the heterogeneous adaptation of the β-cell mass to DXM-induced insulin resistance in rats. The splenic region of the pancreas appears to be particularly responsive to changes in insulin resistance compared to the body and head region that show no adaptation after 6 weeks of DXM treatment.

In line with previous studies we observe that DXM-treatment is associated with insulin resistance and compensatory growth of the β-cell mass (9–13). We now show for the first time that glucocorticoid-induced β-cell adaptation is primarily occurring in the splenic region of the pancreas. Functional and morphological differences of islets derived from different regions of the pancreas have been described (18–20). We have recently shown that high-fat diet (HFD) induced insulin resistance leads to heterogeneous β cell adaptation in mice (14). Remarkably, this adaptation was also most prominent in the splenic region of the pancreas. Together these studies strongly point to the splenic region of the pancreas being involved in the first line of β-cell adaptation to insulin resistance in rodents.

The results of the present study could either be explained by a difference in the local islet environment between different regions leading to differential stimuli for β-cell adaptation and/

or β-cell responses or an intrinsic difference in the capacity of β-cell mass adaptation between islets in the different regions of the pancreas. We have shown before that transplantation of islets isolated from the three regions to an extrapancreatic location in diabetic mice led to a similar compensatory response (14). Therefore, extrinsic factors present in the islet microenvironment may be responsible for the observed topological heterogeneity in β-cell adaptation.

Islets have an extensive vascular network to enable efficient secretion of insulin into the circulation. They receive about 20-times more arterial blood compared to the exocrine pancreas in rats (21, 22). Intravital microscopy and *in vivo* labeling studies have shown that insulin resistance leads to a greater blood flow in islets that is associated with improved β-cell function and proliferation (23, 24). Furthermore, islets are densely innervated by the autonomic nervous system (25) and it has been reported that β-cell mass adaptation can be regulated through neuronal signals from the liver (26). Also, the surrounding exocrine pancreas has a close functional interaction with islets (29) and is known to be topologically heterogeneous (30). Whether the local islet environment plays a role in topologically heterogeneous β-cell adaptation remains to be established.

In human subjects, heterogeneity of the β-cell area throughout the pancreas is well known. Several studies have reported a higher islet density in the tail-region of the pancreas (2, 31, 32). Also, the β-cell mass has been reported to be increased in subjects with obesity (1, 2, 33, 34). However, most studies in humans rely on tissue sampling from the tail-region of the pancreas only (1, 33, 34). Importantly, our study implies that this may not be representative for the entire organ.

The presence of enlarged β-cell clusters in the splenic region in the absence of β-cell hypertrophy in DXM-treated rats points to β-cell proliferation as the major compensatory mechanism in this study. We did not observe an increase in β-cell proliferation at 3 or 6 weeks of DXM treatment. In non-human primates 3 weeks of glucocorticoid treatment was associated with an increase in β-cell proliferation (9). Previous studies in rats show an increased β-cell proliferation already after 3 days of DXM treatment (12, 13, 16). Also in HFD-induced insulin resistance in mice it has been reported that β-cell proliferation begins within the first 7 days of HFD exposure (17). This suggests that the peak in β-cell proliferation induced by DXM-treatment in our study occurred within the first 3 weeks of DXM treatment.

In conclusion, we show that DXM-induced insulin resistance in rats is associated with β-cell adaptation that is topologically heterogeneous throughout the pancreas. Comparison of regional differences may lead to identification of novel mechanisms involved in β-cell adaptation.

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Chapter 4

Topologically heterogeneous β- and α-cell adaptation with maintenance of α- to β-cell ratio in obesity

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Submitted

Abstract

Background/Objectives

In order to maintain glucose homeostasis the number and/or function of insulin-producing pancreatic β-cells can change. It is unknown whether β-cell adaptation is homogeneous throughout the pancreas of human subjects. Hyperglucagonemia is present in type 2 diabetes, but data are lacking whether the glucagon-producing α-cells adapt to changes in weight. In this study we examined β- and α-cell mass of non-diabetic obese and lean human subjects throughout different regions of the pancreas.

Subjects/Methods

Pancreatic tissue of the head-, body- and tail-region of the pancreas was examined from 15 obese organ donors with a Body Mass Index (BMI) ≥ 27 kg/m^2 and age-matched lean organ donors with a BMI ≤ 25 kg/m^2. β- or α-cells were identified by immunostaining for insulin and glucagon, respectively.

Results

In obese subjects β- and α-cell mass were proportionally increased compared to lean subjects (β-cell mass 2.4±0.3 g (obese) vs. 1.6±0.2 g (lean), $p<0.05$; α-cell mass 1.2±0.2 g (obese) vs. 0.8±0.1 g (lean), $p<0.05$), thereby maintaining the α- to β-cell ratio. While the fractional β- and α-cell area were the highest in the pancreatic tail, the homeostatic adaptation to obesity occurred preferentially in the head of the pancreas.

Conclusions

In obese subjects β- and α-cell mass are increased and this adaptation is topologically heterogeneous. As data so far have been derived from studying β- and α-cell mass in tissues from the tail-region of the pancreas, the homeostatic adaptive capacity of humans to obesity has previously been underestimated.

Introduction

Both type 1 and type 2 diabetes are characterized by the loss of functional pancreatic β-cell mass (1). For these patients therapies are needed that restore, maintain or prevent loss of functional β-cells. Therefore, it is critical to understand how the β-cell mass is regulated. In order to maintain glucose homeostasis the number and/or function of insulin-producing pancreatic β-cells can change. In human subjects it is well established that obesity and pregnancy are associated with an increased β-cell mass (2–9). However, several of these studies rely on tissue sampling from the pancreatic tail-region of the pancreas only (4, 5, 7, 9). We have recently shown that high-fat diet induced insulin resistance leads to topologically heterogeneous β-cell adaptation in mice, that is most prominent in the splenic region of the pancreas (10). In human subjects it is not known whether β-cell adaptation is homogeneous throughout the pancreas of human subjects.
Type 2 diabetes is also characterized by hyperglucagonemia and associated with an increased α- to β-cell ratio (11). In two separate studies we have recently shown that in addition to the β-cell mass, the glucagon-producing α-cell mass can also be modulated by dietary changes in mice (12, 13). It is unknown whether obesity modulates the α-cell mass and if so, how this affects the α- to β-cell ratio. In this study we examine the α- and β-cell mass in obese subjects and compare these to the findings in lean subjects as an indication of β- and α-cell mass adaptation to obesity.

Materials and methods

Subjects

Human pancreata were procured through a multiorgan donor program. Pancreatic tissue was used in our study if the tissue could not be used for clinical islet transplantation, according to national laws, and if research consent was present. Pancreatic tissue of the head-, body- and tail-region of the pancreas was examined from obese organ donors with a Body Mass Index (BMI) ≥ 27 kg/m^2 (n=15) and age-matched lean organ donors with a BMI ≤ 25 kg/m^2 (n=15). None of the organ donors had a clinical history of diabetes. Characteristics of the studied subjects are given in supplementary table 1.

Immunohistochemistry

Pancreas samples were fixed overnight in 4% formaldehyde (Klinipath, Duiven, The Netherlands), embedded in paraffin and sliced into 4 µm sections. Pancreatic polypeptide positive cells were identified in the head-regions of the pancreas using rabbit anti-PP IgG (Millipore, Billerica, MA, USA) for 30 min followed by horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h. Sections were developed with 3,3'-diaminobenzidine tetrahydrochloride (DAB) and counterstained with haematoxylin. If microscopic examination revealed a PP-cell rich area the head-region of this pancreas was excluded from the analysis.

For identification of β-cells pancreas sections were immunostained with rabbit anti-insulin IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or guinea pig anti-insulin IgG (Millipore) for 1 h followed by HRP- or alkaline phosphatase-conjugated secondary antibodies for 1 h. α-Cells were identified by immunostaining using rabbit anti-glucagon IgG (Vector Laboratories, Burlingame, CA, USA) for 1 h followed by a HRP-conjugated secondary antibody for 1 h. To identify proliferating β- or alpha-cells, sections were double stained with mouse anti-Ki67 (Becton Dickinson, Franklin Lakes, NJ) and primary antibodies against insulin or glucagon, respectively, overnight at 4°C after heat-induced antigen retrieval in 0.01 M citrate buffer followed by HRP- and AP-conjugated secondary antibodies. Sections were developed with DAB or liquid permanent red (LPR, Dako, Denmark) and counterstained with haematoxylin. Stained sections were digitally imaged (Panoramic MIDI, 3DHISTECH, Budapest, Hungary).

Morphometry

For determining the fractional β-cell area, the percentage of insulin-DAB stained area out of total pancreas area stained with hematoxylin was determined using an image analysis program (Stacks 2.1, LUMC, (10)), excluding large blood vessels, larger ducts, adipose tissue and lymph nodes. The surface area of each insulin-positive cell cluster was measured and used to calculate the average β-cell cluster area per pancreatic region. Islet density was determined by dividing the number of β-cell clusters by the (regional) area that was analyzed. For measurements in the entire organ, the average of the three regions was calculated. The pancreas weight was estimated by use of an equation based on the population data from our own institute (supplemental figure 1, formula: pancreas weight = 2.31 x BMI + 47.7). β-Cell mass was determined by the β-cell area multiplied by the estimated pancreas weight. The fractional α-cell area was determined by calculating the ratio of α-cell area to β-cell area of 20 randomly selected islets per pancreatic region, using Stacks 2.1. α-Cell mass was calculated by multiplying the α- to β-cell ratio by the β-cell mass.

Statistical analysis

Data are presented as means ± SE. Statistical calculations were carried out using GraphPad Prism 5 (GraphPad Software, San Diego, CA). The statistical significance of differences was determined by Mann-Whitney test, or two-way ANOVA, followed by Bonferroni' s multiple-comparisons test, as indicated. Correlations were tested using Spearman rank analysis. $P < 0.05$ was considered statistically significant.

Results

Increased β- and α-cell mass in obese subjects

The average age of obese and lean subjects was similar (53.2±3.3 years (lean) vs. 52.4±3.0 years (obese), p=0.86; table S1). The average body mass index (BMI) was 30.1±0.6 kg/m^2 for obese and 22.5±0.3 kg/m^2 for lean subjects. The β-cell mass was determined by analysing 22.1±1.4 mm^2 pancreatic tissue per donor. The β-cell mass was ~1.5x higher in obese compared to lean subjects (Fig. 1A-C). No significant difference in β-cell cluster area was observed (Fig. 1D) whereas a tendency (p = 0.06) for a higher islet density was present in obese subjects (Fig. 1E). A positive correlation was found between β-cell mass and BMI (Fig. 1F, r=0.32, p<0.05).

α-Cell mass was found to be 50% increased in obese subjects (Fig. 1A, B and G). A similar α- to β-cell ratio was observed in lean and obese subjects (Fig. 1H) resulting in a strong positive correlation between β- and α-cell mass (Fig. 1I). No correlation was found between age and β-cell mass (r=0.19, p=0.16) or α-cell mass (r=-0.28, p=0.08) but a negative correlation was found between age and the α- to β-cell ratio (Fig. 2, r=0.55, p<0.01).

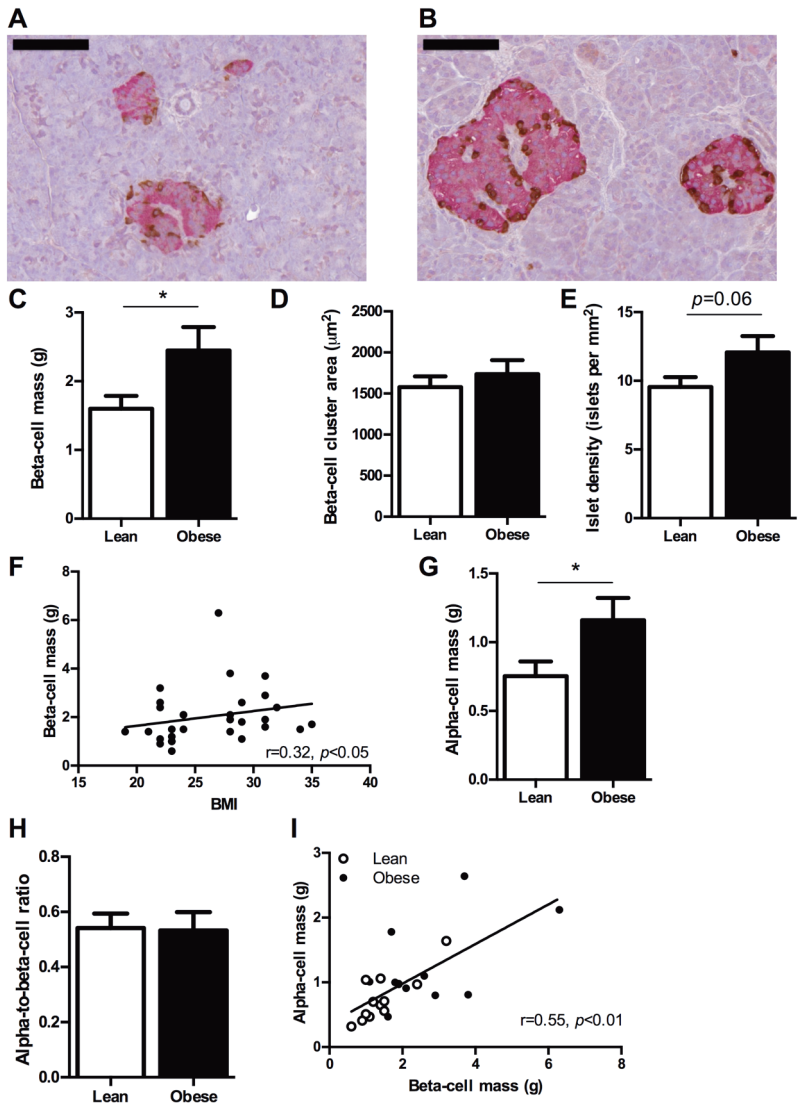


Fig. 1. β- And α-cell mass in lean and obese subjects. A. Representative picture of β-cells (red) and α-cells (brown) in islets of a lean subject. Scale bar = 100 μm. B. Representative picture of β-cells (red) and α-cells (brown) in islets of an obese subject. Scale bar = 100 μm. C. β-Cell mass (n = 15). D. β-Cell cluster area (n = 30). E. Islet density (n = 30). F. Correlation between β-cell mass and BMI (n = 30). G. α-Cell mass (n = 12-14). H. α- to β-cell ratio (n = 12-14). I. Correlation between β- and α-cell mass (n = 26). *p<0.05 by Mann-Whitney U test.

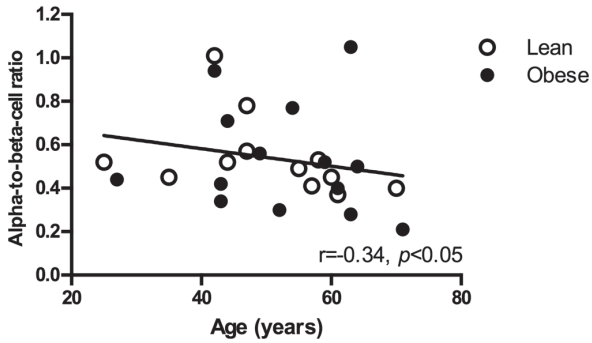


Fig. 2. Correlation between age and α- to β-cell ratio (n = 26).

β- And α-cell area are topologically heterogeneous throughout the pancreas

To assess the distribution of the β- and α-cells throughout the pancreas, the fractional β- or α cell area, islet density, islet size and α- to β-cell ratio were determined by pancreatic region (i.e. head, body and tail). In the tail-region of the pancreas both β- and α-cell area were significantly higher compared to the head and body region in lean subjects (Fig. 3A and D). Islet density was similar throughout the pancreas in lean individuals (Fig. 3B) whereas β-cell cluster area was significantly higher in the tail-region of the pancreas compared to the head-region in lean donors (Fig. 3C). The α- to β-cell ratio was similar throughout the pancreas (Fig. 3E).

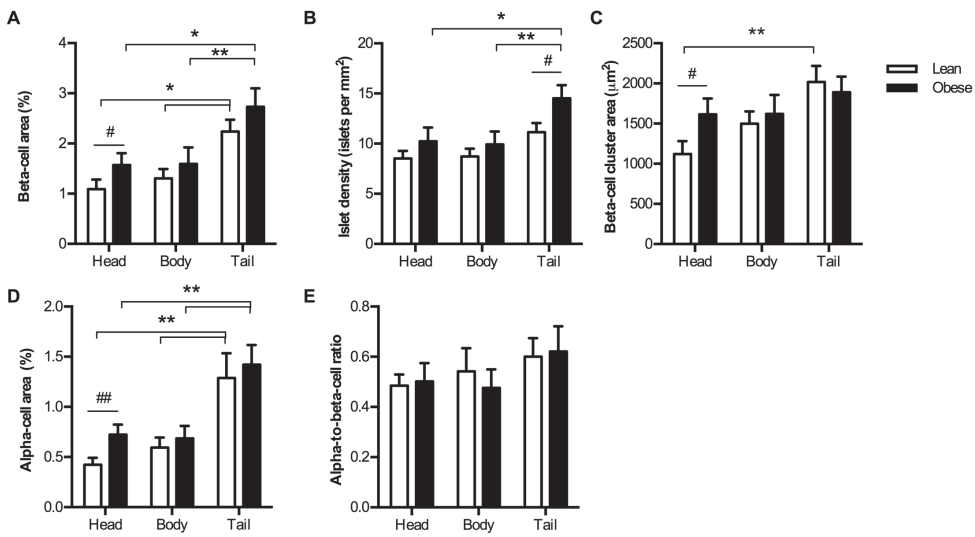


Fig 3. β- And α-cell area in lean and obese subjects. A. Fractional β-cell area by pancreatic region (n = 10-15 per region). B. Islet density by pancreatic region (n = 10-15). C. Mean β-cell cluster area by pancreatic region (n = 10-15). D. Fractional α-cell area by pancreatic region (n = 10-14). E. α- to β-cell ratio by pancreatic region (n = 10-14). *p<0.05 and **p<0.01 by two-way ANOVA followed by Bonferroni's multiple comparisons test, #p<0.05 and ##p<0.01 by Mann-Whitney U test.

β- And α-cell adaptation are topologically heterogeneous in obese human subjects

In obese subjects, islet density was increased in the tail-region of the pancreas (Fig. 3B). When comparing regional islet cell areas between lean and obese individuals, the β-cell area was found to be ~1.4x increased in the head-region of obese subjects (Fig. 3A). This was associated with a significantly increased average β-cell cluster area (Fig. 3C). Also, the α-cell area was ~1.7x increased in the head-region of obese subjects compared to lean controls (Fig. 3D). β-Cell and α-cell proliferation were rarely observed (data not shown). The α- to β-cell ratio throughout the pancreas was similar between lean and obese individuals (Fig. 3E).

Discussion

The main results of our study indicate that obesity is associated with adaptation of both β-and α cell mass and that this adaptation is topologically heterogeneous between different regions in the pancreas.

In line with previous studies (3–7), we found that obesity in humans is associated with an increase of the β-cell mass, most likely to compensate for the increased demand for insulin (14, 15). Also, a strong positive correlation between β- and α-cell mass was observed, which is in accordance by the study of Henquin and Rahier (11). We now show for the first time that the α-cell mass is also increased in obese subjects. Recently it was found that in overweight insulin-resistant non-human primates the fractional α-cell area was significantly increased, and that the changes in α-cell area preceded changes in β-cell area (16). Whether an increased α-cell mass during obesity is a physiological adaptive response to maintain an adequate hormonal balance between insulin and glucagon or that this increase predisposes obese individuals for the development of type 2 diabetes remains an open question (17).

Heterogeneity of the β-cell area throughout the pancreas is well known (6, 18, 19) and in line with these studies we also observe the highest β-cell area in the tail-region of the pancreas. Reers et al. (19) noted an increased islet density in the tail-region when analysing 20 donors with BMIs ranging from 17.2 to 33 kg/m^2. Here we show that this increased islet density is present in obese donors only. Previous studies have observed fewer α-cells in the part of the pancreas that originates from the ventral bud during embryonic development (20, 21). We now show that, within the part of the human pancreas that is derived from the dorsal bud, the α-cell area is higher in the tail-region.

We recently demonstrated that high-fat diet induced insulin resistance leads to topologically heterogeneous β-cell adaptation in the pancreas of mice (10). Now we show that also in human pancreas β- and α-cell adaptation are topologically heterogeneous.

Interestingly, both β- and α-cell area were increased in the head-region of the pancreas in obese compared to lean subjects. Recently, Wang et al. (22) observed a preferential loss of β-cells in the

pancreatic head-region of patients with type 2 diabetes compared to healthy controls. Together these data suggest that preservation of the endocrine cell mass in the head-region of the pancreas may be of importance for maintenance of normoglycemia in humans. In most histological studies of α and/or β-cell adaptation the head-region of the human pancreas was not included (4–7, 11), which may have led to an underestimation of actual changes in these studies.

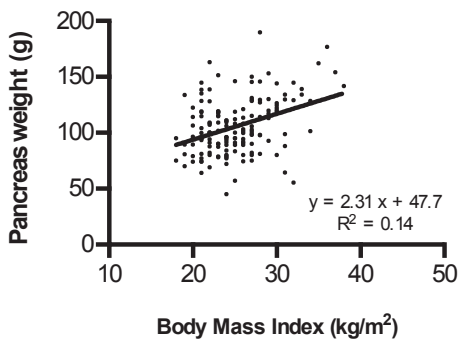
The α- to β-cell ratio was found to be negatively correlated with age. This confirms the observation by Rahier et al. (11) that the negative correlation between α-cell mass and age was stronger than for β-cell mass and age in humans. Furthermore, Saisho et al. (5) showed that ageing in humans was not related to loss of β-cell mass. Together these results indicate that with advanced age the β-cell mass is more constant than the α-cell mass. Whether this is a physiological adaptation to counteract the age-associated deterioration in glucose tolerance, which is associated with increased glucagon concentrations (23), remains to be determined.

Following adaptation of the β- and α-cell mass in obesity, the α- to β-cell ratio was preserved. This is in line with the study by Rahier et al. (11) who found a similar α-to β-cell ratio when comparing subjects with a BMI lower or higher than 25 kg/m^2. We now show that this ratio is also similar throughout different pancreatic regions in both obese and lean subjects. It has been described that human islets have a unique architecture in which heterologous contacts between β- and α-cells are preferred (24). Insulin secretion from individual human β-cells is enhanced when they are coupled to an α-cell (25), possibly due to paracrine cholinergic stimuli secreted by α-cells (26). Altogether, our data show that both β- and α-cell mass are increased in obese subjects and that this adaptation is topologically heterogeneous. The α- to β-cell ratio is similar throughout the pancreas and preserved following adaptation in non-diabetic obese subjects.

Supplemental data

	Lean (BMI<24)				Obese (BMI>27)			
	#	Age (years)	BMI (kg/m2)	F/M	#	Age (years)	BMI (kg/m^2)	F/M
	1	25	22	M	1	27	28	M
	2	35	21	M	2	42	29	M
	3	42	23	F	3	43	29	M
	4	44	23	F	4	43	27	M
	5	47	19	F	5	44	31	F
	6	47	23	F	6	49	29	M
	7	55	24	F	7	51	32	M
	8	57	22	F	8	52	31	F
	9	58	23	F	9	54	28	F
	10	60	22	F	10	59	31	M
	11	61	23	M	11	61	34	M
	12	64	24	M	12	63	35	M
	13	65	24	M	13	63	31	F
	14	68	22	M	14	64	28	F
	15	70	22	M	15	71	28	F
AVG		**53,2**	**22,5**	**7M, 8F**		**52,4**	**30,1**	**9M, 6F**
SEM		3,3	0,3			3,0	0,6	

Supplemental table 1. Characteristics of pancreas donors.



Supplemental figure 1.
Relationship between body mass index and pancreas weight of 154 pancreas donors.

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Chapter 5

Glucagon like peptide-1 receptor agonist treatment reduces β-cell mass in normoglycaemic mice

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Abstract

Aims/hypothesis

Incretin-based therapies improve glycaemic control in patients with type 2 diabetes. In animal models of diabetes, glucagon-like peptide-1 receptor agonists (GLP-1RAs) increase β-cell mass. GLP-1RAs are also evaluated in non-diabetic individuals with obesity and cardiovascular disease. However, their effect on β-cell mass in normoglycaemic conditions is not clear. Here, we investigate the effects of the GLP-1RA liraglutide on β-cell mass and function in normoglycaemic mice.

Methods

C57BL/6J mice were treated with the GLP-1RA liraglutide or PBS and fed a control or high-fat diet (HFD) for 1 or 6 weeks. Glucose and insulin tolerance tests were performed after 6 weeks. BrdU was given to label proliferating cells 1 week before the animals were killed. The pancreas was taken for either histology or islet isolation followed by a glucose-induced insulin-secretion test.

Results

Treatment with liraglutide for 6 weeks led to increased insulin sensitivity and attenuation of HFD-induced insulin resistance. A reduction in β-cell mass was observed in liraglutide-treated control and HFD-fed mice at 6 weeks, and was associated with a lower β-cell proliferation rate after 1 week of treatment. A similar reduction in α-cell mass occurred, resulting in an unchanged α- to β-cell ratio. In contrast, acinar cell proliferation was increased. Finally, islets isolated from liraglutide-treated control mice had enhanced glucose-induced insulin secretion.

Conclusions/interpretation

Our data show that GLP-1RA treatment in normoglycaemic mice leads to increases in insulin sensitivity and β-cell function that are associated with reduced β-cell mass to maintain normoglycaemia.

Introduction

Glucagon-like peptide 1 (GLP-1) is an incretin hormone secreted by intestinal L cells in response to ingestion of carbohydrates and lipids (1). Activation of the GLP-1 receptor (GLP-1R) on pancreatic β-cells leads to glucose-dependent insulin secretion and improves glycaemic control in patients with type 2 diabetes (2, 3). In animal models of diabetes these therapies increase β-cell mass (4–6).

The β-cell mass is tightly controlled in order to keep glucose levels within a narrow range. When the demand for insulin is chronically increased by physiological or pathological changes, such as pregnancy or obesity, there is an increase in β-cell function and/or β-cell mass (7, 8). When the demand for insulin decreases, for example postpartum, the β-cell mass reverts to its original capacity (9).

Besides their effects on β-cells, GLP-1R agonists (GLP-1RAs) exert several extrapancreatic effects that may be of therapeutic benefit. GLP-1RAs decrease body weight and are associated with reduced blood pressure, improved lipid profiles and improved endothelial function in patients with type 2 diabetes (10, 11). Therefore, these compounds have also been evaluated in non-diabetic individuals with obesity or cardiovascular disease (12–15). However, the effect of GLP-1RAs on β-cells in these normoglycaemic conditions is not clear. Therefore, we investigated the effects of GLP-1RA treatment on β-cell mass and function in normoglycaemic mice.

Methods

Animals

Male C57BL/6J mice, 8–9 weeks old (Charles River Laboratories, Wilmington, MA, USA), were housed under standard conditions with a 12 h light/dark cycle and free access to food and water. Mice were fed a normal diet (control; 10% of total energy intake derived from lard fat, 16.3 kJ [3.9 kcal]/g; D12450B, Research Diets, New Brunswick, NJ, USA) or a high-fat diet (HFD; 45% of total energy intake derived from lard fat, 19.7 kJ [4.7 kcal]/g; D12451, Research Diets) for 1 or 6 weeks. Average food intake was determined weekly per cage housing three or four mice. Liraglutide (0.1 mg/kg, Novo Nordisk, Bagsvaerd, Denmark) or PBS was given twice daily with at least a 10 h interval between subcutaneous injections. Treatment was discontinued 1 day before the animals were killed. Body weight was determined after overnight fasting. Animal experiments were approved by the institutional ethical committee on animal care and experimentation at the Leiden University Medical Center.

Glucose and insulin tolerance tests

Insulin tolerance and glucose tolerance were assessed after 6 weeks of treatment. An intraperitoneal insulin tolerance test (ITT) was performed in animals that had been fasted for 6 h. After measuring basal blood glucose concentration from the tail vein, 0.75 U/kg insulin was injected followed by monitoring of the blood glucose concentrations after 15, 30 and 60 min. An intraperitoneal glucose tolerance test (GTT) was performed in overnight-fasted mice. Blood samples were drawn from the tail vein before injecting 2 g/kg glucose and after 15, 30, 60 and 120 min. Blood glucose concentrations were measured using a glucose meter (Accu-Chek, Roche, Basel, Switzerland) and insulin concentrations were measured by ELISA (Chrystal Chem, Downers Grove, IL, USA). Plasma IL-6, IL-1β and monocyte chemoattractant protein-1 (MCP-1) were detected using a custom cytokine/metabolic multiplex assay (Meso Scale Discovery, Gaithersburg, MD, USA).

Pancreas dissection and islet isolation

Mice were anaesthetised by isoflurane inhalation and exsanguinated. The pancreases were dissected and weighed. For immunohistochemistry the pancreas was fixed by immersion in a 4% (vol./vol.) paraformaldehyde solution. For islet isolation the pancreases of six to eight mice were pooled and digested using 3 mg/ml collagenase (Sigma-Aldrich, St Louis, CA, USA) in RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 2 μg/ml DNAse I (Pulmozyme, Roche) and shaken at 37°C for 15–18 min until a homogeneous digest was obtained. The digest was then washed three times with cold RPMI medium supplemented with 10% (vol./vol.) heat-inactivated FCS (Bodinco, Alkmaar, the Netherlands) and penicillin/streptomycin (100 U/ml and 100 μg/ml, respectively; Invitrogen). Islets were purified from exocrine tissue by manual selection picking under a dissecting microscope.

Glucose-induced insulin secretion test

A glucose-induced insulin secretion test was performed on freshly isolated islets. Groups of ten islets were incubated in a modified Krebs-Ringer bicarbonate buffer with HEPES (KRBH) containing 115 mmol/l NaCl, 5 mmol/l KCl, 24 mmol/l NaHCO3, 2.2 mmol/l CaCl2, 1 mmol/l MgCl2, 20 mmol/l HEPES and 2 g/l human serum albumin (Cealb, Sanquin, the Netherlands), pH 7.4. Islets were washed and pre-incubated with KRBH buffer containing 2 mmol/l glucose for 1.5 h at 37°C. They were then incubated in 2 mmol/l glucose KRBH buffer for 1 h at 37°C and switched to 20 mmol/l glucose KRBH buffer for 1 h at 37°C. Supernatant fractions were kept for determination of insulin concentration by ELISA (Mercodia, Uppsala, Sweden). Islet cells were lysed by sonication in distilled water. Islet insulin content was measured by acid ethanol extraction followed by ELISA (Mercodia). Islet DNA content was determined by Quant-iT PicoGreen dsDNA kit (Invitrogen).

Immunohistochemistry and morphometry

In order to obtain representative samples of the entire organ, pancreases from each mouse (six per group) were cut into three pieces (duodenal, gastric and splenic region) (16) that were embedded in paraffin blocks and sliced into 4 µm sections. For each analysis two to four sections per block, with an interval of at least 200 µm between sections, were immunostained and analysed. The average of the three regions was taken as a measure for the entire organ.

For the identification of β-cells, nine to twelve sections per mouse pancreas were immunostained with guinea pig anti-insulin IgG (Millipore, Billerica, MA, USA) or rabbit anti-insulin IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h followed by horseradish peroxidase (HRP)- or alkaline phosphatase-conjugated secondary antibodies for 1 h. α-Cells were identified by immunostaining by rabbit anti-glucagon IgG (Vector Laboratories, Burlingame, CA, USA) for 1 h followed by HRP-conjugated secondary antibody for 1 h. Sections were developed with 3,3′-diaminobenzidine tetrahydrochloride (DAB) or Liquid Permanent Red (LPR; Dako, Glostrup, Denmark) and counterstained with haematoxylin. Stained sections were digitally imaged (Panoramic MIDI, 3DHISTECH, Budapest, Hungary).

β-cell and pancreas areas stained with haematoxylin were determined using an image-analysis program (Stacks 2.1, LUMC, Leiden, the Netherlands), excluding large blood vessels, larger ducts, adipose tissue and lymph nodes as previously described (16). β-Cell mass was determined by the ratio of β-cell area to pancreas area multiplied by the pancreas weight. β-Cell cluster area was determined as the average area of β-cell clusters (defined as ≥4 β-cells per cluster) per mouse. α-Cell mass was determined by calculating the ratio of α-cell area to β-cell area per islet, using ImageJ software (ImageJ, US National Institutes of Health, Bethesda, MD, USA), multiplied by the β-cell mass.

To label proliferating β-cells, mice were given 50 mg/kg BrdU (Sigma-Aldrich) subcutaneously twice daily during the entire period for the 1 week study and the final 7 days for the 6 week study. Sections were double stained for insulin-LPR and BrdU (BrdU staining kit, Invitrogen). Stained sections were digitally imaged (Panoramic MIDI). BrdU-positive β-cells were assessed as a proportion of all β-cells. Pancreatic duct cells were identified based on their typical morphology and location. The number of BrdU-positive duct cells was counted. The number of BrdU-positive acinar cells was counted using Stacks 2.1 and expressed as a percentage of the total number of acinar cells. The area in which these were counted was divided by the total number of cells as a measure of acinar cell size. Apoptotic β-cells were identified by the TUNELtechnique (Roche) in combination with insulin immunostaining and were counted. The investigator was blind to the experimental conditions during counting.

Statistical analysis

Data are presented as mean±SEM. Statistical calculations were carried out using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). The statistical significance of differences was determined by two-way ANOVA, followed by Bonferroni's multiple comparisons test, as appropriate. $p < 0.05$ was considered statistically significant.

Results

Metabolic characteristics of control and HFD-fed mice following 6 weeks of liraglutide treatment

Liraglutide treatment for 6 weeks was associated with decreased body weight and increased insulin sensitivity in both control and HFD-fed mice (Fig. 1a–c). After a glucose load, liraglutide treatment attenuated the peak glucose concentration induced by HFD at 30 min (Fig. 1d), but this did not reach significance for the AUC for glucose (Fig. 1e). In mice on a normal diet there was no significant difference in glucose concentrations after liraglutide treatment (Fig. 1d). In both liraglutide-treated groups the early response of insulin secretion during the GTT was increased to a similar extent as in HFD-fed mice (Fig. 1f, g).

Increased acinar cell proliferation after liraglutide treatment

After 6 weeks, pancreatic weight was significantly greater in liraglutide-treated mice (Fig. 2a), despite the decrease in body weight (Fig. 1a). Therefore, we analysed the effect of liraglutide treatment on the exocrine pancreas. Treatment with liraglutide was associated with an increased size of acinar cells in control mice (Fig. 2b). We counted 13,441 ± 439 acinar cells per mouse. The number of proliferating acinar cells was 65% higher in liraglutide-treated control mice (Fig. 2c, d). A similar effect was observed in liraglutide-treated HFD-fed mice, though the difference was less prominent. No significant difference in the number of proliferating duct cells was observed between the groups after 6 weeks of treatment (528 ± 28 duct cells were counted per mouse; Fig. 2e, f). Assessment of pro-inflammatory cytokine plasma concentrations (IL-1β, IL-6 and MCP-1) showed no significant differences between the groups (electronic supplementary material [ESM] Fig. 1).

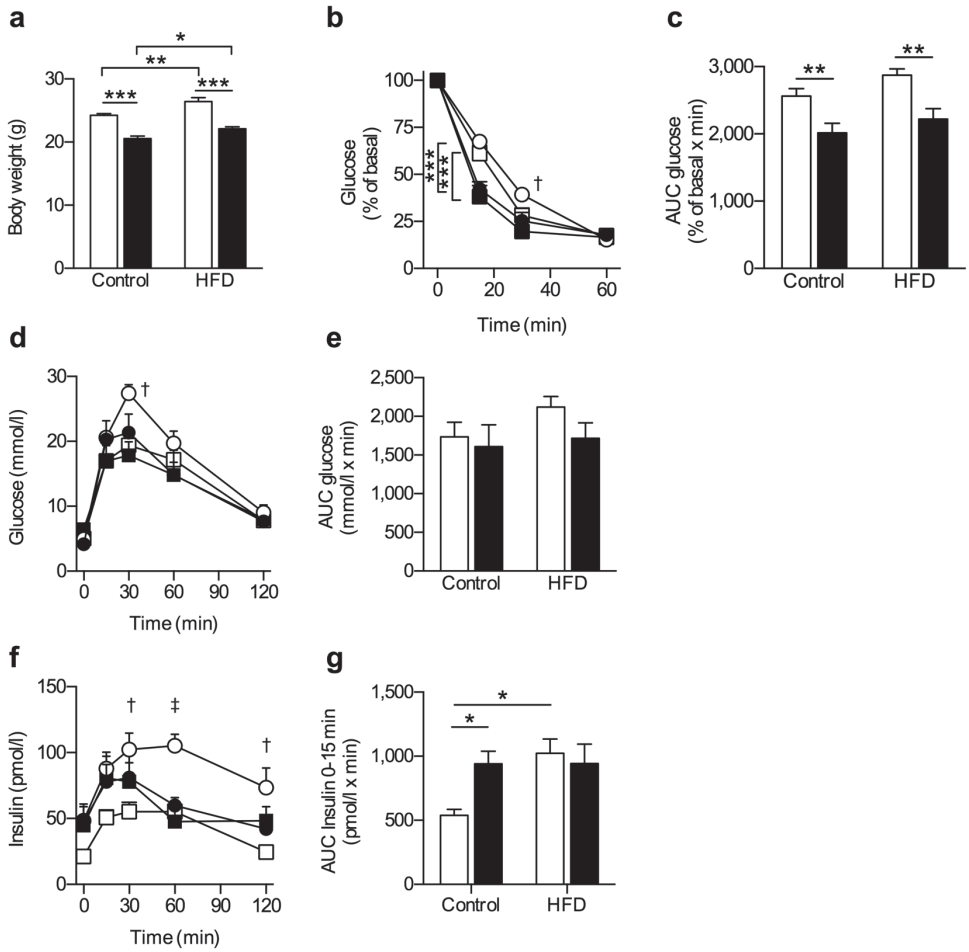


Fig. 1. Metabolic characteristics of control (squares) and HFD-fed (circles) mice treated with liraglutide (black) or PBS (white) for 6 weeks. (**a**) Body weight ($n = 13$–14 mice). (**b**) Blood glucose concentrations expressed as percentage of basal glucose concentration during the ITT ($n = 7$–8 mice). (**c**) AUC of glucose concentrations during the ITT corrected for basal glucose concentration ($n = 7$–8 mice). (**d**) Blood glucose concentrations during GTT ($n = 6$ mice). (**e**) AUC for blood glucose concentrations during the GTT ($n = 6$ mice). (**f**) Insulin concentrations during GTT ($n = 5$–6 mice). (**g**) AUC 0–15 min insulin concentrations during GTT ($n = 5$–6 mice). *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$; † $p < 0.05$ vs control + PBS; ‡ $p < 0.05$ vs control + PBS and HFD + liraglutide.

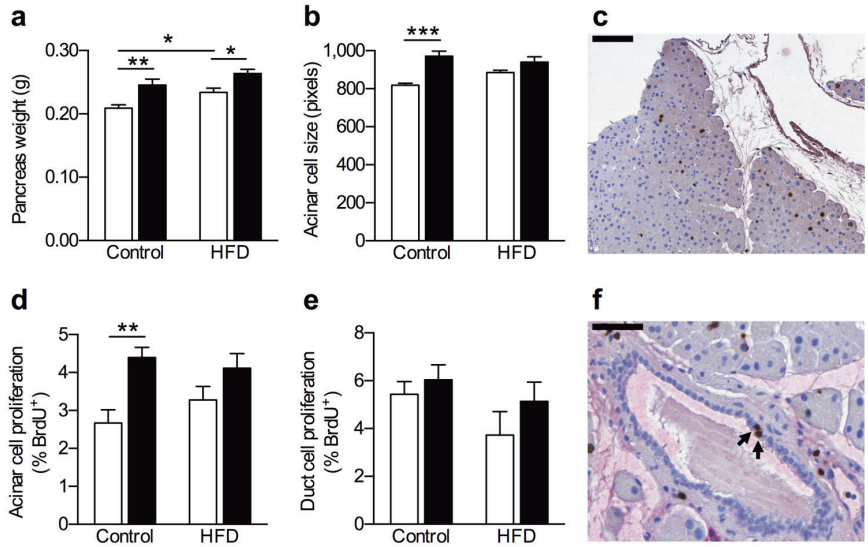


Fig. 2. The effects of liraglutide (black bars) or PBS (white bars) treatment on the exocrine pancreas in control and HFD-fed mice after 6 weeks. (**a**) Pancreas weight ($n = 13–14$ mice). (**b**) Acinar cell size ($n = 6$ mice). (**c**) Representative picture of proliferating acinar cells, BrdU (brown); scale bar, 100 µm. (**d**) Acinar cell proliferation, BrdU labelling during the final 7 days ($n = 6$ mice). (**e**) Duct cell proliferation, BrdU labelling during the final 7 days ($n = 6$ mice). (**f**) Representative picture of proliferating duct cells (arrows), BrdU (brown); scale bar, 50 µm. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

Liraglutide reduces β-cell and α-cell mass

β-cell mass, determined by analysing 94.9 ± 4.7 mm2 pancreatic tissue per mouse, was reduced in control and HFD-fed mice after treatment with liraglutide for 6 weeks (Fig. 3a). This was associated with a decreased average β-cell cluster area (Fig. 3b). Liraglutide treatment did not affect insulin content in freshly isolated islets (Fig. 3c). No difference in the number of apoptotic β-cells was found between groups after counting 1,129 ± 170 β-cells per mouse (Fig. 3d). To determine the number of proliferating β-cells 1,807 ± 128 cells per mouse were counted. Liraglutide treatment in both control and HFD-fed mice was associated with a lower number of proliferating β-cells after 1 week, but after 6 weeks no difference was observed (Fig. 3e–g). Similarly, the α-cell mass was reduced in liraglutide-treated control mice. The lower α-cell mass in HFD-fed mice was unaffected by liraglutide treatment (Fig. 3h). The ratio of α- to β-cell area did not change significantly between the groups (Fig. 3i).

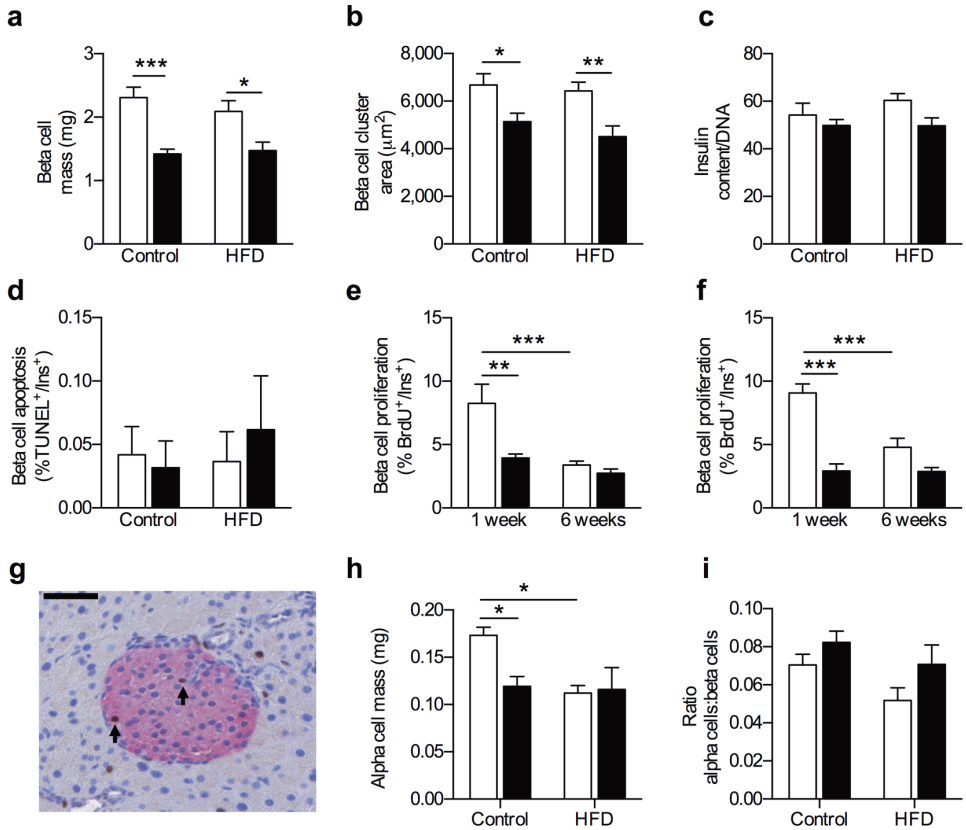


Fig. 3. β-Cell and α-cell mass in control and HFD-fed mice after liraglutide (black bars) or PBS (white bars) treatment. (**a**) β-Cell mass after 6 weeks of treatment ($n=6$ mice). (**b**) β-Cell cluster area after 6 weeks of treatment ($n=6$ mice). (**c** β-cells Insulin content from isolated islets corrected for DNA content ($n=24$). (**d**) β-Cell apoptosis, identified by TUNEL⁺/insulin⁺ staining, after 6 weeks of treatment ($n=6$). (**e**) β-Cell proliferation in control mice after 1 and 6 weeks of treatment, BrdU labelling during the final 7 days ($n=6$ mice). (**f**) β-Cell proliferation in HFD-fed mice after 1 and 6 weeks of treatment, BrdU labelling during the final 7 days ($n=6$ mice). (**g**) Representative picture of proliferating β-cells (arrows), BrdU (brown) and insulin (red). Scale bar = 50 µm. (**h**) α-Cell mass after 6 weeks of treatment ($n=6$ mice). (**i**) Ratio of α-cell area to β-cell area after 6 weeks of treatment ($n=6$ mice). *$p<0.05$, **$p<0.01$ and ***$p<0.001$.

Increased glucose-induced insulin release from isolated islets of liraglutide-treated control mice

Finally, in order to investigate whether 6 weeks of liraglutide treatment had specific effects on β-cell function in the presence of a reduced β-cell mass, we assessed glucose-induced insulin secretion in isolated islets. Basal insulin secretion (2 mmol/l glucose) was increased twofold in islets from liraglutide-treated control mice (Fig. 4a). Glucose stimulation of islets from liraglutide-treated control mice resulted in a 35% increase in insulin secretion (Fig. 4a). As expected, glucose stimulation of islets from HFD-fed mice led to increased insulin secretion compared with mice fed

regular chow (Fig. 4b). This glucose stimulation was unchanged in HFD-fed mice that had been treated with liraglutide for 6 weeks (Fig. 4b).

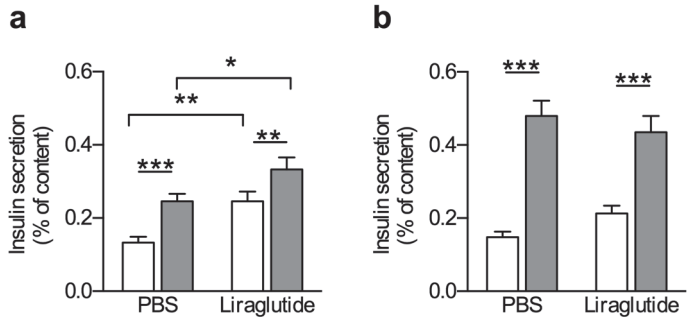


Fig. 4. Glucose-induced insulin secretion from isolated islets of control mice and HFD-fed mice treated with liraglutide for 6 weeks. Insulin secretion is presented as a percentage of total insulin content. (**a**) Insulin secretion of islets from control mice, $n=24$, and (**b**) insulin secretion of islets from HFD-fed mice, $n=23–24$; white bars, 2 mmol/l glucose; grey bars, 20 mmol/l glucose. *$p<0.05$, **$p<0.01$ and ***$p<0.001$.

Discussion

The effect of GLP-1-based therapy on insulin secretion from β-cells has been reported to be glucose dependent (17–19), but its effect on β-cell mass under different glycaemic conditions is less clear. While GLP-1RA treatment increases β-cell mass in animal models of diabetes, we now show a reduction in β-cell mass in normoglycaemic mice.

GLP1-RA treatment of non-diabetic obese individuals results in weight loss and improved β-cell function (12, 13). Therefore, it is relevant to understand how β-cell mass adapts during GLP1-RA treatment under normoglycaemic conditions and different dietary situations. So far, few studies have investigated the effect of GLP-1RA in non-diabetic animals and showed either no difference or an increase in β-cell proliferation after short-term treatment for between 2 and 10 days (6, 20–22).

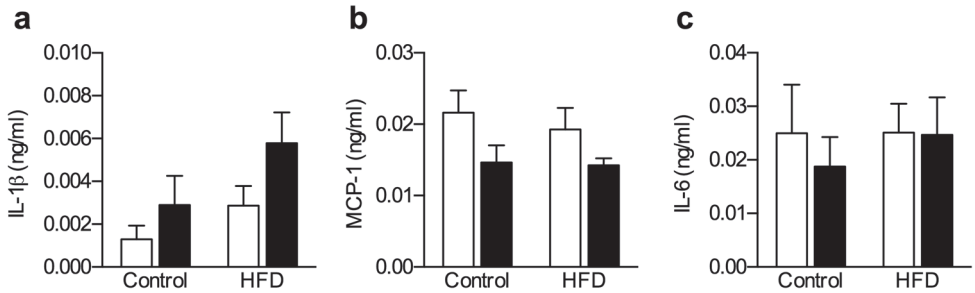
Liraglutide treatment for 6 weeks resulted in decreased body weight and increased insulin sensitivity in normoglycaemic mice and HFD-fed mice, in line with earlier studies (23, 24). In addition, the increased early response of insulin secretion during the GTT in liraglutide-treated mice is in line with the working mechanism of GLP-1 (25, 26). This was associated with a major reduction in β-cell mass in both control and HFD-fed mice. The lower β-cell proliferation rate that we observed in mice treated for 1 week suggests that the β-cell mass adapted rapidly after the start of liraglutide treatment. Interestingly, we also show that α-cell mass was reduced to such an extent that the ratio of α- to β-cells remained unchanged.

Acute GLP-1R stimulation of β-cells is known to increase insulin secretion in a glucose-dependent manner (17–19). We show that sustained GLP-1RA treatment during normoglycaemic conditions is associated with increased insulin secretion from isolated islets even in the absence of direct GLP-1RA stimulation *in vitro*. In HFD-fed mice, liraglutide treatment increased insulin sensitivity, but the enhanced insulin secretory response remained. Together these data suggest that liraglutide treatment in normoglycaemic mice leads to increased insulin sensitivity and an enhanced insulin secretory response from existing β-cells, thereby reducing the need for new β-cells. In addition, these data imply that chronic GLP-1RA treatment during normoglycaemia results in an increased β-cell function as was shown in non-diabetic obese individuals (12). In contrast, GLP-1RA treatment does not increase β-cell proliferation during normoglycaemia.

Finally, we observed increased acinar cell proliferation after liraglutide treatment indicating that this effect can occur during normoglycaemia. There was a non-significant difference in duct cell proliferation after 6 weeks of treatment. These observations and the findings of other studies (27–32) raise the issue of whether GLP-1-based therapies are a potential risk for pancreatitis; some studies did not observe this effect (33, 34), however, which may reflect the animal model used, the age of the animals and/or the labelling and counting methods used for proliferating cells. In our study, there was no indication of a systemic inflammatory state. Ductal proliferation is also associated with the development of pancreatic adenocarcinoma but the relationship between GLP-1-based treatment and the development of new pancreatic malignant lesions is not clear (35).

In conclusion, our data indicate that GLP-1RA under normoglycaemic conditions can have different effects on pancreatic islet and non-islet cells. GLP-1RA treatment during normoglycaemia results in a reduction in β-cell mass, whereas it exerts proliferative effects on the exocrine pancreas.

Supplementary material



ESM Fig. 1. The effect of liraglutide (black bars) or PBS (white bars) treatment on pro-inflammatory cytokines after 6 weeks. (**a**) Plasma interleukin-1β (IL-1β) concentrations. (**b**) Plasma monocyte chemoattractant protein-1 (MCP-1) concentrations. (**c**) Plasma interleukin-6 (IL-6) concentrations. HFD = high-fat diet.

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Chapter 6

Long-term ketogenic diet causes glucose intolerance and reduced β- and α-cell mass but no weight loss in mice

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Abstract

High-fat, low-carbohydrate ketogenic diets (KD) are used for weight loss and for treatment of refractory epilepsy. Recently, short-time studies in rodents have shown that, besides their beneficial effect on body weight, KD lead to glucose intolerance and insulin resistance. However, the long-term effects on pancreatic endocrine cells are unknown. In this study we investigate the effects of long-term KD on glucose tolerance and β- and α-cell mass in mice. Despite an initial weight loss, KD did not result in weight loss after 22 wk. Plasma markers associated with dyslipidemia and inflammation (cholesterol, triglycerides, leptin, monocyte chemotactic protein-1, IL-1β, and IL-6) were increased, and KD-fed mice showed signs of hepatic steatosis after 22 wk of diet. Long-term KD resulted in glucose intolerance that was associated with insufficient insulin secretion from β-cells. After 22 wk, insulin-stimulated glucose uptake was reduced. A reduction in β-cell mass was observed in KD-fed mice together with an increased number of smaller islets. Also α-cell mass was markedly decreased, resulting in a lower α- to β-cell ratio. Our data show that long-term KD causes dyslipidemia, a proinflammatory state, signs of hepatic steatosis, glucose intolerance, and a reduction in β- and α-cell mass, but no weight loss. This indicates that long-term high-fat, low-carbohydrate KD lead to features that are also associated with the metabolic syndrome and an increased risk for type 2 diabetes in humans.

Introduction

High-fat, low-carbohydrate ketogenic diets (KD) are used in weight loss programs and are associated with improvement of the glycemic status in obese subjects (1, 2) and patients with type 2 diabetes (3, 4). KD are also used as an effective treatment for refractory epilepsy (5–7).
Blood glucose levels are tightly controlled by the hormones insulin and glucagon produced by pancreatic β- and α-cells, respectively. When the consumption of carbohydrates is limited, the body switches from a glucose-based energy metabolism to a fat-based metabolism in which β-oxidation of free fatty acids (FFA) serves as the primary source of energy. This leads to a permanent state of ketosis. Insulin counteracts ketogenesis by stimulating the use of glucose as primary energy source and by decreasing the release of FFA in the circulation (8). In contrast, glucagon stimulates ketogenesis, hepatic glucose production, and lipolysis (9, 10). Changes in glucose metabolism are associated with adaptation of the number and/or function of β-cells to produce and secrete an adequate amount of insulin (11, 12). Also α-cell mass can be modulated by dietary changes (13). Inadequate adaptation leads to glucose intolerance and eventually results in diabetes mellitus (8, 14).
Despite their beneficial effects on weight loss and epileptic seizures, KD may have adverse side effects such as kidney stones, impaired growth, osteoporosis, and hyperlipidemia (15, 16) on the long term. Furthermore, several short-term studies in rodents have shown that KD leads to hepatic steatosis, insulin resistance, and glucose intolerance (17–19).
It is unknown whether the metabolic effects induced by long-term KD also affect the endocrine pancreas. In addition, the effect of KD on glucose metabolism has only been studied in mice after short-term diets (18–20). Therefore, we investigated the effects of a long-term KD on glucose tolerance and pancreatic β- and α-cell mass.

Materials and methods

Animals

Male C57BL/6J mice, 10 wk old (Charles River Laboratories, Wilmington, MA), were fed a KD (Research Diets, New Brunswick, NJ) or regular chow (control; Special Diets Services, Essex, UK) for 22 wk. The proportion of calories derived from nutrients for the KD, which is similar to other studies (18–21), and control diet is described in Table 1. In addition, 8-wk-old male C57BL/6J mice were fed a KD or a normal diet (D12450B; Research Diets) for 1 wk. Before euthanization, mice were anesthetized by isoflurane inhalation. Animal experiments were approved by the ethical committee on animal care and experimentation of the Leiden University Medical Center.

	Control		KD	
	kcal/100kcal	g/100 g	kcal/100 kcal	g/ 100 g
Protein	27.2	22.5	5.9	10.0
Carbohydrate	61.2	50.4	1.0	2.0
Fat	11.5	4.2	93.1	72.0
Dietary fiber		16.2		8.5
Methionine		0.37		0.29
Choline		0.14		0.34
Saturated fatty acids	1.9	0.7	29.6	22.8
Monounsaturated fatty acids	3.0	1.1	33.7	25.9
Polyunsaturated fatty acids	4.2	1.6	24.6	18.9
ω-3 fatty acids	0.5	0.2	2.0	1.5
Energy density, kcal/g	3.3		6.9	

Table 1. Diet composition. KD, ketogenic diet

Glucose and insulin tolerance test

Glucose tolerance was assessed after 1, 5, 12, and 20 wk of diet. An intraperitoneal glucose tolerance test (GTT) was performed in overnight-fasted mice. Blood samples were drawn from the tail vein before injecting 2 g/kg glucose and after 15, 30, 60, and 120 min. Insulin tolerance was assessed after 22 wk of diet. An intraperitoneal insulin tolerance test (ITT) was performed in animals that had been fasted for 6 h. After measuring basal blood glucose concentration from the tail vein, 1.0 U/kg insulin was injected followed by monitoring of the blood glucose concentrations after 15, 30, 60, and 90 min. Blood glucose concentrations were measured using a glucose meter (Accu Chek, Roche, Basel, Switzerland) and β-hydroxybutyrate concentrations using a ketone meter (Precision Xtra System; Abbot Diabetes Care, Alameda, CA). Insulin concentrations were measured by ELISA (Ultra Sensitive Mouse Insulin ELISA kit; Chrystal Chem, Downers Grove, IL).

Plasma analysis

Plasma leptin, IL-6, IL-1β, and monocyte chemotactic protein (MCP)-1 were detected using a custom Cytokine/Metabolic multiplex assay (Mesoscale Discovery, Gaithersburg, MD). Plasma cholesterol, triglycerides, alanine aminotransferase (ALT), and aspartate aminotransferase (AST) were measured on a Roche Modular P800 analyzer (Roche).

Liver triglyceride analysis

Following euthanization, the liver was dissected, weighed, and stored at –80°C. Lipid extraction was performed using a modified protocol of Bligh and Dyer (22). Briefly, liver tissue was homogenized in ice-cold methanol. Lipids were extracted by addition of ice-cold chloroform. After centrifugation, the supernatant was dried with nitrogen gas. Lipids were dissolved in chloroform with 2% Triton X-100 (Sigma-Aldrich) and dried. Finally, lipids were dissolved in 100 µl H_20. Triglyceride content was measured using an enzymatic kit (Roche), and protein content

was measured using the BCA protein assay kit (Pierce, Rockford, IL). Liver triglyceride content was defined as total triglyceride content per milligram of protein.

Islet morphometry

The pancreas was dissected and weighed after euthanization. To obtain representative samples of the entire organ, pancreata from each mouse (6/group) were cut in three pieces (duodenal, gastric, and splenic region) (23) that were fixed by immersion in a 4% paraformaldehyde solution, embedded in paraffin blocks, and sliced into 4-μm sections. From each block four sections, with an interval of at least 200 μm between sections, were immunostained and analyzed. The average of the three regions was taken as a measure for the entire organ.

For the identification of β-cells, sections were immunostained with guinea pig anti-insulin IgG (Millipore, Billerica, MA) or rabbit anti-insulin IgG (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h followed by horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h. α-Cells were identified by immunostaining by rabbit anti-glucagon IgG (Vector Laboratories, Burlingame, CA) for 1 h followed by HRP-conjugated secondary antibody for 1 h. Sections were developed with 3,3′-diaminobenzidine tetrahydrochloride or liquid permanent red (LPR, Dako, Denmark) and counterstained with hematoxylin. Stained sections were digitally imaged (Panoramic MIDI; 3DHISTECH).

β-Cell area and pancreas area were determined using an image analysis program (Stacks 2.1; LUMC), excluding large blood vessels, larger ducts, adipose tissue, and lymph nodes as previously described (3). β-Cell mass was determined by the ratio of β-cell area to pancreas area multiplied by the pancreas weight. β-Cell cluster size was determined as the median size of β-cell clusters (defined as ≥4 β-cells per cluster) per mice. α-Cell mass was determined as previously described (13) by calculating the ratio of α-cell area to β-cell area, using Image J software (Image J; National Institutes of Health, Bethesda, MD) multiplied by the β-cell mass.

Statistical analysis

Data are presented as means ± SE. Statistical calculations were carried out using GraphPad Prism 5 (GraphPad Software, San Diego, CA). The statistical significance of differences was determined by an unpaired Student's *t*-test, Mann-Whitney test, or two-way ANOVA, followed by Bonferroni's multiple-comparisons test, as appropriate. $P < 0.05$ was considered statistically significant.

Results

No weight loss after 22 wk of KD

The KD was not associated with weight loss after 22 wk of diet, despite an initial weight loss during the first weeks of diet (Fig. 1A). After 22 wk, body weight in the KD-fed mice was 34.8 ± 1.2 vs. 32.2 ± 0.4 g in the control mice ($P < 0.05$). After 5 wk, increased concentrations of

circulating β-hydroxybutyrate were measured and remained elevated after 20 wk of diet, which indicates a ketotic state in KD-fed mice (Fig. 1B).

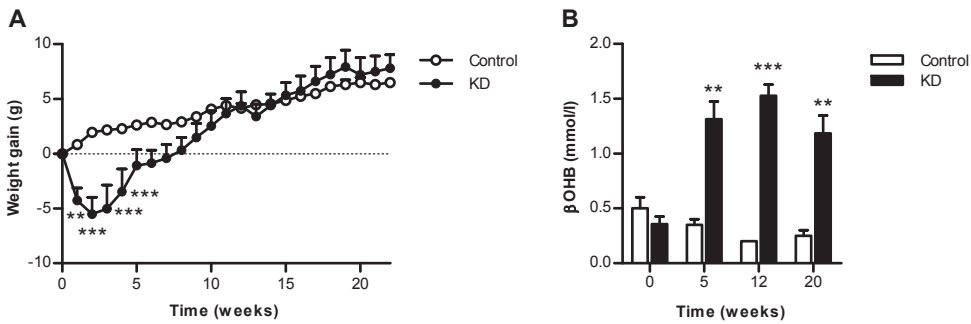

Fig. 1. Effects of 22-wk control or ketogenic diet (KD) on weight gain and ketosis. *A*: weight gain during 22 wk of diet (n = 8–10 mice). *B*: β-hydroxybutyrate (βOHB) levels (n = 2–7 mice). $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. control.

KD leads to dyslipidemia, a proinflammatory state, and hepatic steatosis

To assess the metabolic profile of KD-fed mice, several markers that are also associated with the metabolic syndrome in humans were measured. After 22 wk, there was a significant increase of plasma cholesterol, triglyceride, leptin, MCP-1, IL-1β, and IL-6 concentrations in plasma of KD-fed mice (Table 2). To assess whether these systemic markers were related to metabolic changes in the liver, the intrahepatic triglyceride levels were determined as a measure of hepatic steatosis. Liver triglyceride content was increased twofold after 22 wk of KD [379 ± 41 nmol/mg protein (KD) vs. 159 ± 19 nmol/mg protein (control), $P < 0.01$]. In addition, plasma ALT and AST were significantly increased.

	Control	KD
Total cholesterol, mg/dl	92.7 ± 3.1	141.3 ± 9.5 [b]
Triglyceride, mg/dl	42.0 ± 1.5	64.9 ± 6.8[b]
Leptin, ng/ml	0.63 ± 0.37	2.35 ± 0.99[a]
MCP-1, pg/ml	6.20 ± 0.73	12.65 ± 1.40[c]
IL-1β, pg/ml	1.19 ± 0.31	3.86 ± 1.17[a]
IL-6, pg/ml	3.20 ± 2.11	19.84 ± 8.02[a]
ALT, U/l	31.3 ± 0.9	80.6 ± 15.8 [b]
AST, U/l	89.0 ± 10.7	155.2 ± 31.4 [a]

Table 2. Plasma markers of the metabolic syndrome in control mice and mice fed a ketogenic diet for 22 wk. Values are means ± SE; n = 8-10 mice. MCP, monocyte chemotactic protein; IL, interleukin; ALT, alanine aminotransferase; AST, aspartate aminotransferase. [a]P<0.05, [b]P<0.01, and [c]P<0.001.

Long-term KD leads to glucose intolerance

Glucose tolerance tended to be decreased after 5 wk, but KD-fed mice became markedly glucose intolerant after 12 wk of diet (Fig. 2, A–D). After 1 wk KD, insulin concentrations were increased during the GTT (Fig. 2, E and I). However, continuation of the diet for 5 wk or longer resulted in insufficient insulin secretion from β-cells to maintain glucose tolerance (Fig. 2, F–H and J–L). After 20 wk diet, glucose-induced insulin concentrations were significantly decreased in KD-fed mice (Fig. 2L). Insulin-dependent glucose uptake assessed by an ITT was also reduced in KD-fed mice compared with control mice after 22 wk (Fig. 3, A and B). Also, the fasting insulin-to-glucose ratio was significantly increased in KD-fed mice (Fig. 3C).

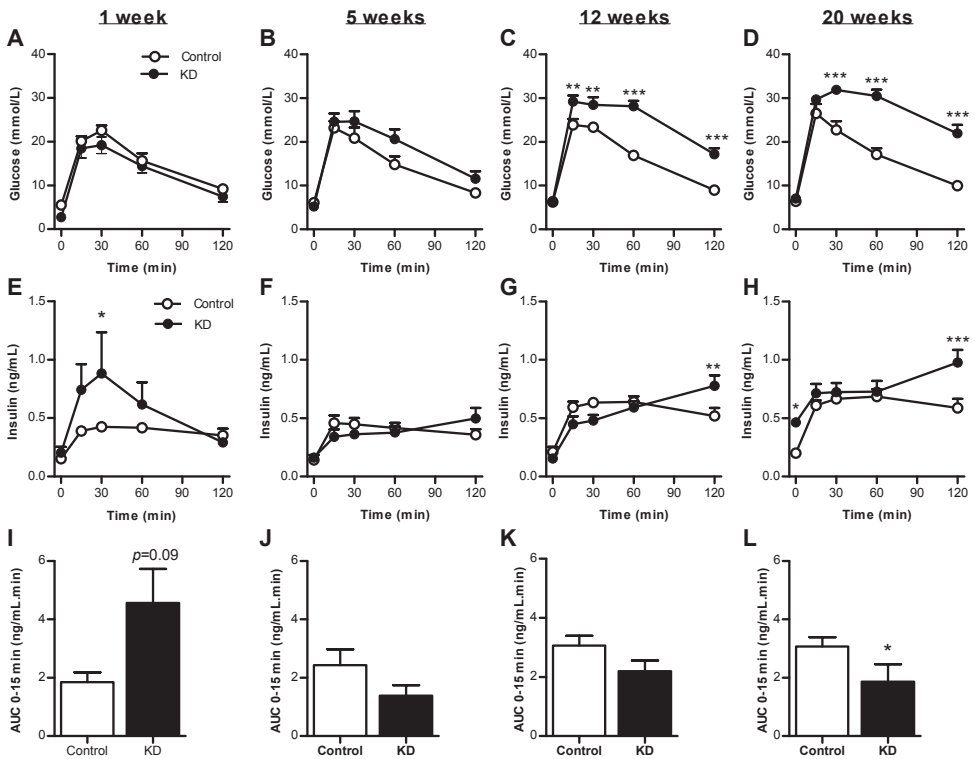

Fig. 2. Glucose tolerance in control and KD-fed mice. Blood glucose concentrations during the glucose tolerance test after 1 (*A*), 5 (*B*), 12 (*C*), and 20 wk (*D*) of diet (n = 5–10 mice). Insulin concentrations during the glucose tolerance test after 1 (*E*), 5 (*F*), 12 (*G*), and 20 (*H*) wk of diet (n = 3–10 mice). Area under the curve (AUC) 0–15 min insulin concentrations during the glucose tolerance test after 1 (*I*), 5 (*J*), 12 (*K*), and 20 (*L*) wk of diet (n = 4–10 mice). *P < 0.05, **P < 0.01, and ***P < 0.001 vs. control.

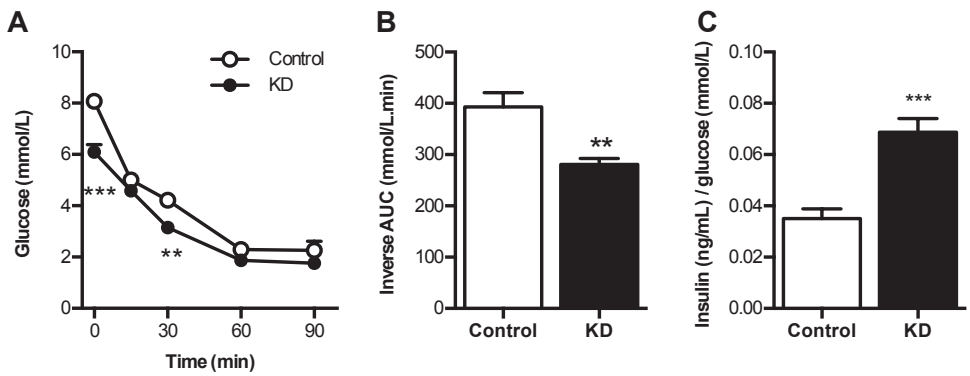

Fig. 3. Insulin tolerance in control and KD-fed mice. *A*: blood glucose concentrations during the insulin tolerance test (n = 7–8 mice) after 22 wk of diet. *B*: inverse AUC below baseline glucose concentrations during the insulin tolerance test (n = 7–8 mice). *C*: fasting insulin-to-glucose ratio after 20 wk of diet (n = 8–9 mice). $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. control.

KD leads to decreased β- and α-cell mass.

After 22 wk, β-cell mass in KD-fed mice, determined by analyzing 175.1 ± 7.1 mm^2 pancreatic tissue/mouse, was decreased (Fig. 4, A–C). The density of islets was unchanged (Fig. 4D), but the median β-cell cluster size was significantly decreased (Fig. 4E). This was because of a relatively increased number of islets with a size smaller than 2,500 μm^2 in KD-fed mice (Fig. 4F). The α-cell mass was reduced by 50% in KD-fed mice after 22 wk, which resulted in a decreased α-cell/β-cell ratio in KD-fed mice (Fig. 5, A–D).

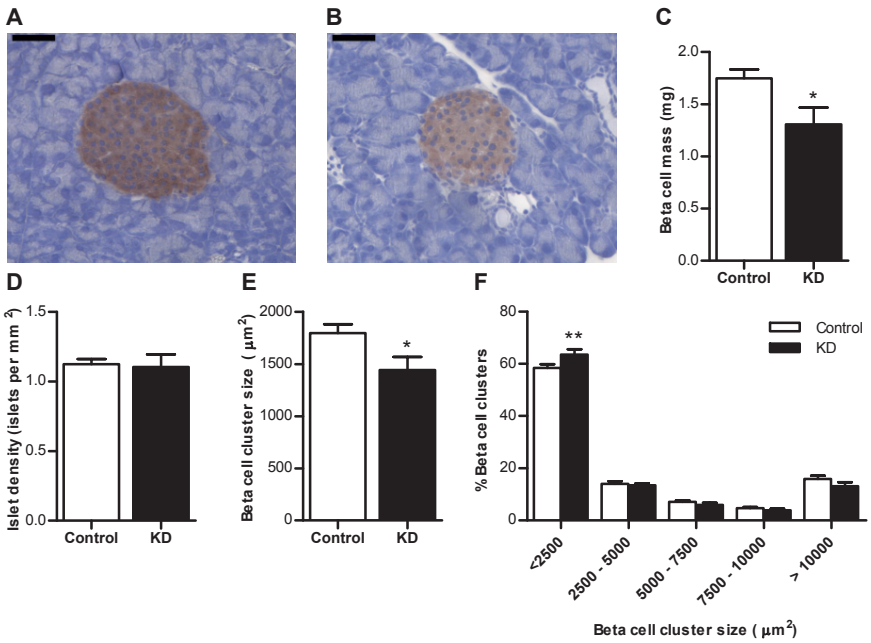

Fig. 4. β-Cell mass in control and KD-fed mice after 22 wk. *A*: representative image of β-cells (brown) in control mice. Scale bar = 50 µm. *B*: representative image of β-cells (brown) in KD-fed mice. Scale bar = 50 µm. *C*: β-cell mass (n = 8–10 mice). *D*: islet density (n = 8–10 mice). *E*: median β-cell cluster size (n = 8–10 mice). *F*: β-cell cluster distribution (n = 8–10 mice). $*P < 0.05$ and $**P < 0.01$ vs. control.

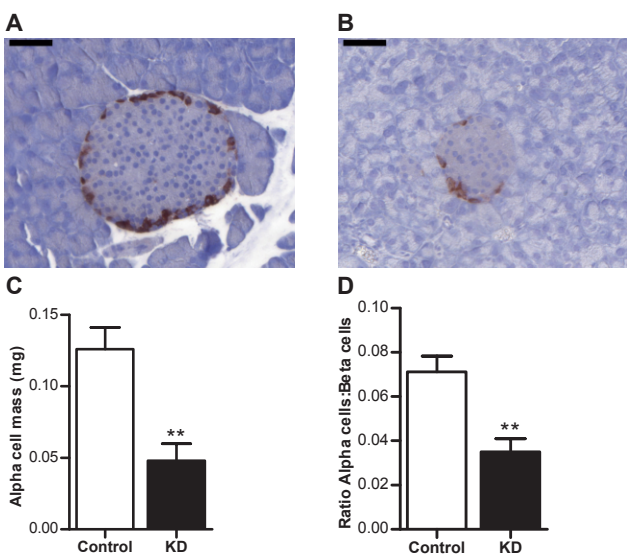

Fig. 5. α-Cell mass in control and KD-fed mice after 22 wk. *A*: representative image of α-cells (brown) in control mice. Scale bar = 50 µm. *B*: representative image of α-cells (brown) in KD-fed mice. Scale bar = 50 µm. *C*: α-cell mass (n = 7–10 mice). *D*: α-cell/β-cell ratio (n = 7–10 mice). $**P < 0.01$ vs. control.

Discussion

High-fat, low-carbohydrate KD have been associated with beneficial effects on body weight and epileptic seizures. However, their effects on pancreatic endocrine cells and glucose metabolism on the long term are less clear. The main results of our study show that long-term KD in mice causes glucose intolerance and a reduction in both β- and α-cell mass, but no weight loss. This indicates that long-term KD leads to features that are also associated with the metabolic syndrome in humans and an increased risk for type 2 diabetes.

KD resulted in weight loss in the first weeks of the diet, which is in line with previous reports in rodents (19–21). Also in patients with refractory epilepsy, short-term (3–4 mo) KD treatment resulted in decreased body weight (24, 25). However, we now show that a prolonged KD is not associated with weight loss. After 12 wk, weight gain is similar in both groups of mice. Long-term use of KD in children with epilepsy resulted in slowed growth but did not change the body mass index (25–27). In rats it has been shown that 4–6 wk KD leads to visceral fat accumulation (28) and increased leptin concentrations (29). We did not assess the effect of long-term KD on body composition. However, the elevated plasma leptin concentrations observed in our study suggest that prolonged KD eventually leads to regaining weight because of an increase of body fat.

Long-term KD leads to increased plasma cholesterol and triglyceride levels indicative of dyslipidemia. In previous short-term studies, increased plasma cholesterol levels were observed after 9 wk of diet (20), whereas at that time point plasma triglyceride levels were lower. Also, in both adults and children with refractory epilepsy, KD was associated with increased plasma cholesterol and triglyceride levels (30, 31). Furthermore, the increased plasma cytokine concentrations in our study suggest a systemic proinflammatory state in long-term KD-fed mice. This is in line with the observation that short-term KD in mice is associated with increased inflammatory markers in liver and adipose tissue (20) and macrophage infiltration in the liver (18). Also, we show that KD-fed mice had increased plasma levels of the chemokine MCP-1, which is associated with increased macrophage recruitment to the liver (32). In patients with the metabolic syndrome, increased MCP-1 is associated with macrophage infiltration in fat tissue and a proinflammatory state (33). Recently, a short-term very-low-carbohydrate diet in overweight and obese humans resulted in increased concentrations of the inflammatory marker C-reactive protein (34), which is associated with an increased risk for the metabolic syndrome (35).

Dyslipidemia and the proinflammatory state induced by KD may be the consequence of the high content of saturated fatty acids. It was shown that a short-term polyunsaturated fat-enriched KD did not adversely alter lipid metabolism in adults (36). Also, supplementation of a high-fat diet with ω-3 polyunsaturated fatty acids has been shown to prevent high-fat diet-induced insulin resistance by reducing inflammasome-dependent inflammation in rodents (37). However, in our study, the higher content of ω-3 fatty acids in the KD could not prevent proinflammatory effects on the long term. Whether further modification of the fatty acid content of KDs can attenuate dyslipidemia and the proinflammatory effects on the long term needs further study.

Furthermore, KD-fed mice showed signs of steatohepatitis as indicated by the increased hepatic triglyceride content and elevated AST and ALT levels. This is in line with metabolic changes observed in previous studies in mice (18–21) and may indicate an early stage of nonalcoholic fatty liver disease (18, 38). Recent short-term studies have shown that supplementation of KD with choline or methionine could limit hepatic steatosis and the proinflammatory state of the liver, respectively (39, 40). However, the similar methionine and higher choline content of KD in our study did not prevent signs of steatohepatitis in the long term. Altogether these data indicate that a prolonged KD leads to dyslipidemia, a proinflammatory state, and signs of hepatic steatosis.

In this study we show that long-term KD is associated with pronounced glucose intolerance and reduced insulin-stimulated glucose uptake. This was also observed in a recent study by Bielohuby et al. after 4 wk of KD in mice (17). We now show that insulin concentrations were increased to maintain normoglycemia after 1 wk of KD, but continuation of the KD resulted in insufficient insulin secretion to maintain glucose tolerance.

This inadequate insulin secretory response could be the result of β-cell dysfunction, an insufficient β-cell number, or a combination of these two mechanisms. The results in this study strongly indicate that long-term KD leads to an insulin secretory defect. Furthermore, not only after short-term diet (17) but also after long-term KD, β-cell mass is reduced. An inadequate function and/or number of β-cells leads to insufficient insulin secretion that results in glucose intolerance and ultimately type 2 diabetes in humans (8, 41). Interestingly, also the α-cell mass was decreased considerably, which is in line with decreased glucagon levels that have been observed after 5 wk of KD in mice (19). In relative terms, this decreased α-cell mass was even more pronounced than the reduction in β-cell mass, resulting in a major decrease of the α-cell/β-cell ratio. Decreased glucagon levels lead to less gluconeogenesis from the liver, which may prevent hyperglycemia in KD-fed mice. Whether this change in α-cell mass is a direct consequence of KD or a response to counteract glucose intolerance remains to be elucidated.

Altogether, the results of this study indicate that a long-term high-fat, low-carbohydrate KD in mice does not cause weight loss and leads to glucose intolerance and a reduction in both β- and α-cell mass. In addition, dyslipidemia, a proinflammatory state, and signs of hepatic steatosis are observed. Effects of short-term diets cannot be automatically translated to metabolic effects after long-term diet use.

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Chapter 7

A high-throughput screening platform using primary human islets to assess β-cell function

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Submitted

Abstract

Aims

β-Cell dysfunction plays a crucial role in all types of diabetes mellitus. Therapies that restore β-cell mass and/or function are needed. High-throughput platforms using primary human β-cells can be a powerful tool for the screening of viral-delivered shRNA or small compound libraries to identify new mechanisms involved in β-cell dysfunction and to identify possible starting points for therapeutic interventions. We developed a high-throughput culture platform for primary human islets to assess β-cell function.

Methods

Three culture systems were established in microwell plates format and compared: intact human islets, and islet cells cultured either in monolayer on ECM or reaggregated into islet-cell clusters. β-Cell function was determined by measuring glucose-induced insulin secretion and responsiveness to known insulin secretagogues. In addition, we assessed the efficiency of adeno- or lentivirus mediated transduction of the cells.

Results

All three culture platforms were set up successfully over a period of 3 days. Glucose-induced insulin secretion of islet cell aggregates was similar to intact islets, whereas the response of islet cells in monolayer was reduced. Exposure to glucagon-like peptide-1 receptor agonists did not enhance insulin secretion, independent of the culture platform used. Activation of cAMP/EPAC-2 signal transduction and inhibition of K-channels enhanced glucose-induced insulin secretion in intact islets and islet cell aggregates, but not in monolayer culture. In contrast to intact islets, dispersed islet cells were efficiently transduced with adeno- or lentivirus.

Conclusions

We present three culture platforms in microwell format using primary human islet cells in which β-cell function can be assessed. Dispersed islet cells can be efficiently transduced using adeno- or lentivirus. Intact islets and islet cell aggregates did respond to insulin secretagogues, while islet cells in monolayer did not. These platforms can be used for high-throughput screening of viral-delivered shRNA or small compound libraries to identify new mechanisms involved in β-cell function and survival.

Introduction

Diabetes mellitus affects 382 million people worldwide and by 2035 this is expected to increase to 592 million people (1). β-Cell dysfunction plays a crucial role in all types of diabetes. Despite intensive treatment with diets and/or current antihyperglycemic agents, normalization of glycemic control can often not be achieved and patients are at risk to develop long-term micro- and macrovascular complications (2). Therefore, novel therapies are needed that restore β-cell function.

High-throughput screenings of viral-delivered shRNA or small compound libraries can be used to identify novel mechanisms involved in the regulation of glucose-induced insulin secretion. However, the development of robust high-throughput assay platforms of intact islets is challenging because islet sizes are heterogeneous and the virus transduction efficiency of these three-dimensional cell clusters is low (3).

Currently no assay platforms are present in which human islet function is assessed. Existing assay platforms are mostly using rodent-derived cell lines and are set up to assess insulin gene expression or protein content (4–7). Following glucose stimulation only a fraction of the total insulin content is secreted from β-cells, which makes these existing read-outs poor indicators of secretory function (8). Therefore, the aim of this study is to set up a high-throughput culture system using primary human islets in which β-cell function can be assessed. Three different culture systems were compared: intact human islets, and cells from dispersed human islets cultured either in monolayer or reaggregated into islet-cell clusters.

Materials and Methods

Human islet isolation and cell culture

Human pancreata were procured through a multiorgan donor program. Islet isolation was performed in the Good Manufacturing Practice facility of our institute according to the method described by Ricordi et al. (9). Isolated islets could be used for scientific research if the number and/or quality of the islets were insufficient for clinical islet transplantation, in accordance with national laws, and if research consent was available. Alternatively islets were obtained from Asterand (Detroit, MI, USA) or Tissue Solutions (Glasgow, UK). Islet purity was determined by dithizone staining. Only islet fractions with a purity of >70% were used in the studies. Islets were cultured in CMRL 1066 medium (Cellgro; Mediatech, Manassas, VA, USA) supplemented with 10% heat-inactivated FBS, 2 mmol/l L-glutamine, 10 mmol/l HEPES, 1.2 mg/ml nicotinamide, 50 µg/ml Gentamycin and penicillin/streptomycin (50 U/ml and 50 ug/ml respectively).

Islet dissociation and culture

Islets were dispersed into single cells by incubation with TrypLE Express (Invitrogen; Thermo Fisher Scientific, Waltham, MA, USA) at 37°C while gently pipetting up and down for 8 – 12 minutes. For monolayer culture 384-wells plates (Greiner Bio-One, Frickenhausen, Germany) were coated with a mixture (referred to as ECM) of 50% (vol/vol) ECM (final concentration 0.5 mg/ml; Sigma-Aldrich, St Louis, CA, USA), 37.5% PureCol (final concentration 1.12 mg/ml; Inamed, Gauting, Germany), 5% DMEM 10x (Gibco; Thermo Fisher Scientific), 5% $NaCO_3$ (37 g/l), 2.5% Hepes (1 M), pH 7.4. Coated plates were incubated at 37°C for at least 30 minutes. Per 384-well 10,000 islet cells were plated. For the aggregate culture 20,000 cells were seeded in 96-wells U-shaped ultra-low binding plates (Costar, Corning, New York, USA). In parallel, intact islets were cultured at a density of 20 islets per well in 96-wells U-shaped ultra-low binding plates (Costar).

Virus transduction

Virus transduction was performed 2 hours after seeding the cells. Adenoviruses expressing the cDNA of the Zsgreen fluorescent protein, or the shRNA adenoviral construct expressing an shRNA sequence with no significant homology to any known mammalian genes (eGFP) or an empty virus vector were produced as described before (10). Islet cells cultured in monolayer and islet cell aggregates were transduced by the addition of adenovirus suspension resulting in a final MOI of 2 infectious particles per cell. Lentivirus transductions of islet cells were performed using vectors derived from pRRL – cPPT-CMV-GFP-PRE. Third generation self-activating lentivirus vectors were produced as described before (11). Islet cells cultured in monolayer and islet cell aggregates were transduced by the addition of lentivirus resulting in a final MOI of 2 (3). After 24 hours, the proteins Zsgreen protein was visualised using fluorescence microscopy and the transduction efficiency was determined by estimating the percentage of cells expressing the fluorescent protein of at least 3 wells per donor.

Glucose-stimulated insulin secretion

Three days after seeding the cells or islets a glucose-induced insulin secretion test was performed. Intact islets and islet cell aggregates were transferred to a 96-wells transwell plate (Corning). Cells were washed two times with a modified Krebs-Ringer Bicarbonate HEPES (KRBH) buffer containing 115 mM NaCl, 5 mM KCl, 24 mM $NaHCO_3$, 2.2 mM $CaCl_2$, 1 mM $MgCl_2$, 20 mM HEPES and 2 g/l human serum albumin, pH 7.4 (incubation buffer). Cells were primed for 1.5 h at 37 °C in incubation buffer supplemented with 2 mM glucose (low glucose). Subsequently, the cells were incubated in fresh incubation buffer supplemented with 2 mM glucose for 1 h to measure the basal insulin secretion levels. The cells were then incubated for 1 h in incubation buffer supplemented with 16.7 mM glucose (high glucose) in the presence or absence of insulin secretagogues. Insulin secretagogues tested were liraglutide (Novo Nordisk, Bagsvaerd, Denmark), exendin-4 (Sigma-Aldrich), the EPAC-2 agonist Reh3 (kindly provided by H. Rehmann),

forskolin (Sigma-Aldrich) and 3-isobutyl-1-methylxanthine (IBMX; Calbiochem, Millipore, Billerica, MA, USA), and tetraethylammonium chloride (TEA; Sigma-Aldrich). Insulin concentrations were determined by ELISA (Mercodia, Uppsala, Sweden) in the supernatants that were collected after low and high glucose incubation. For each donor n=4 wells per condition were tested, from which the average was calculated. The stimulation index of insulin was expressed as the ratio of insulin secreted during high glucose over low glucose. The stimulation index of insulin secretagogues was expressed as the ratio of insulin secreted during high glucose in the presence of the insulin secretagogue over high glucose.

Immunofluorescence staining

Cells were fixed in 4% paraformaldehyde for 30 minutes and washed in PBS. Intact islets or islet cell aggregates were spun down at high speed in fluid agar. Agar-containing cell pellets were embedded in paraffin blocks and sliced into 4 µm sections. For identification of β-cells, sections were incubated with guinea-pig-anti-insulin IgG (1:200, Millipore) and for the identification of α-cells with rabbit-anti-glucagon IgG (1:200, Vector Laboratories, Burlingame, CA, USA) for 1 hour, followed by secondary antibodies TRITC-anti-guinea pig (1:400, Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and goat-anti-rabbit Alexa-488 (1:500, Molecular Probes; Thermo Fisher Scientific) and Dapi (Vector Laboratories) was used as a nuclear staining. For islet cells cultured in monolayer, cells were permeabilized with 0.5% Triton for 10 minutes after which the cells were incubated with mouse-anti-c-peptide IgG (1:1000, Millipore) and rabbit-anti-glucagon IgG (1:100, Vector Laboratories) overnight. Hoechst was used as nuclear staining and secondary antibodies were goat-anti-mouse Alexa-568 (1:1000, Molecular Probes) and goat-anti-rabbit Alexa-488 (1:500, Molecular Probes).

Statistical analysis

Data are expressed as means ± SEM. Statistical calculations were carried out using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). The statistical significance of differences was determined by Mann-Whitney test or Kruskal-Wallis test, followed by Dunn's multiple comparison test, as appropriate. $P<0.05$ was considered statistically significant.

Results

Cellular composition of human islet cells cultured either in monolayer or in 3D aggregates is similar to intact islets

Human islets were obtained from pancreas of 20 organ donors (13 M / 7 F, age 47.8±2.5 years, Body Mass Index 25.9±0.7 kg/m^2). The purity of the islets was 85.6+/-1.3% (range 80 - 95%) as determined by dithizone staining (Fig. 1A). Intact human islets were dispersed into single

cells which were cultured either in monolayer on a matrix of ECM (Fig. 1B) or reaggregated islet cell clusters (Fig. 1C). For the monolayer culture different matrices were tested (gelatin, poly-l-lysin, methylcellulose, collagen-I, laminin, ECM (data not shown). Using ECM, cells were firmly attached to the matrix, which was essential for the multiple washing steps that are required for the glucose-induced insulin secretion tests. For islet cell aggregates, spontaneous reaggregation of 20,000 islet cells resulted in one islet cell cluster without affecting cell viability (data not shown) that could be easily transferred to trans-well membrane plates for the glucose-induced insulin secretion test. To determine the percentage of β- or α-cells present in the three culture platforms, the presence of insulin or glucagon immunostaining was examined in 324±73 (islets), 265±5 (monolayer) and 371±50 (aggregates) cells per donor. After 3 days of culture the percentage of β- or α-cells present in islet cells cultured either in monolayer or in islet cell aggregates was similar to intact human islets (Fig. 1D-G).

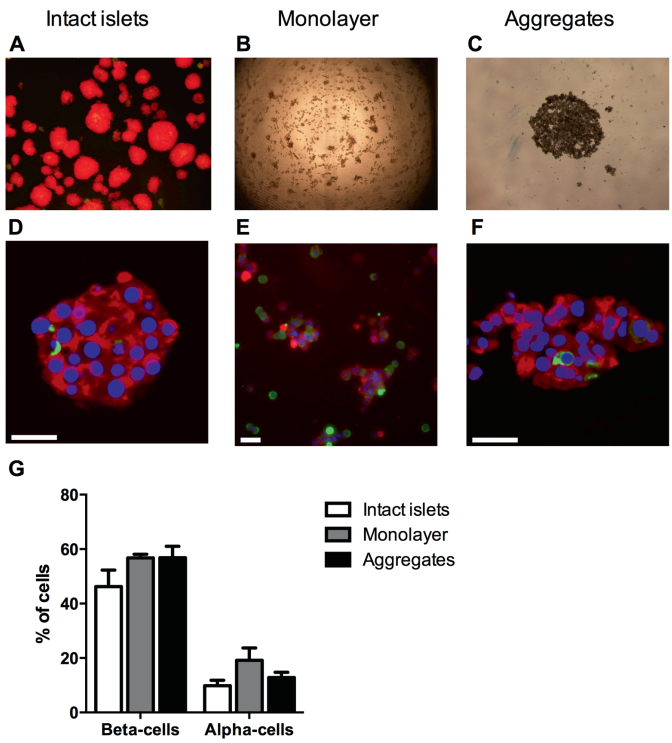

Figure 1. Morphologic appearance and cellular composition of intact human islets, islet cells in monolayer and islet cell aggregates. A. Purified human islets after isolation stained for insulin using dithizone (red), 40x magnification. B. Islet cells cultured in monolayer on ECM, 40x magnification. C. Re-aggregated islet cells, 40x magnification. D-F. Representative pictures of an intact human islet (D), islet cells in monolayer (E) and aggregated islet cells (F), immunostained for insulin (red) and glucagon (green) to identify β-cells and α-cells, respectively. Cell nuclei are stained using DAPI/Hoechst (blue), scale bar = 20 μm. G. Percentage of β-cells and α-cells of total cell number of intact human islets (n=5 donors), islet cells in monolayer (n=2 donors) or islet cell aggregates (n=5 donors).

Efficient virus transduction in human islet cells in monolayer or islet cell aggregates

The transduction efficiency of cells from dispersed human islets was assessed by expression of adenoviral-transduced Zsgreen. Zaldumbide et al. (3) have shown that in intact human islets, primarily the outer layer of islet cells is transduced by virus. For islet cells in monolayer a transduction efficiency of >80% was reached using adenovirus with MOI 2 (Fig 2A). Also, after transduction of single cells in suspension, islet cells were able to cluster together into islet cell aggregates resulting in a transduction efficiency with adenovirus of >80% with MOI 2 (Fig 2B). Similar results were obtained using lentivirus (data not shown).

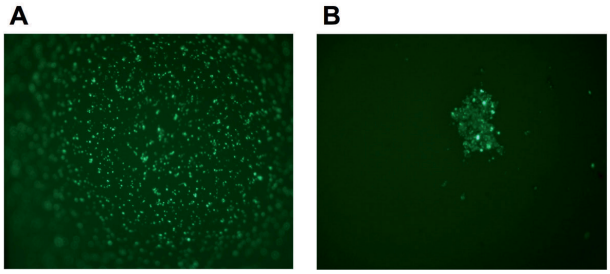

Figure 2. Transduction efficiency of islet cells in monolayer and islet cell aggregates. A. Representative pictures of islet cells in monolayer and B. aggregated islet cells, transduced with adenovirus expressing Zsgreen (MOI 2) imaged 24 hours post-transduction, 40x magnification.

Cultured intact islets and islet cell aggregates maintain glucose-induced insulin secretory response

Next we assessed the insulin secretory response of human islet cells in the different culture platforms. Intact human islets cultured in a 96-well plate preserved their glucose-induced insulin secretory response after 3 days (Fig. 3A). High glucose stimulation of intact islets resulted in a ~8x increase of insulin secretion compared to low glucose (Fig. 3B, E). Although glucose stimulation resulted in increased insulin secretion from islet cells cultured in monolayer, this response was significantly lower than in intact islets (Fig. 3C, E). In contrast, the stimulation index of islet cells cultured in aggregates was similar to intact human islets (Fig. 3D, E). The stimulation index was not significantly affected by adenovirus transduction of islet cells cultured either in monolayer or aggregates after 3 days (Fig. 3F). Similar results were obtained using lentivirus (data not shown and (3)).

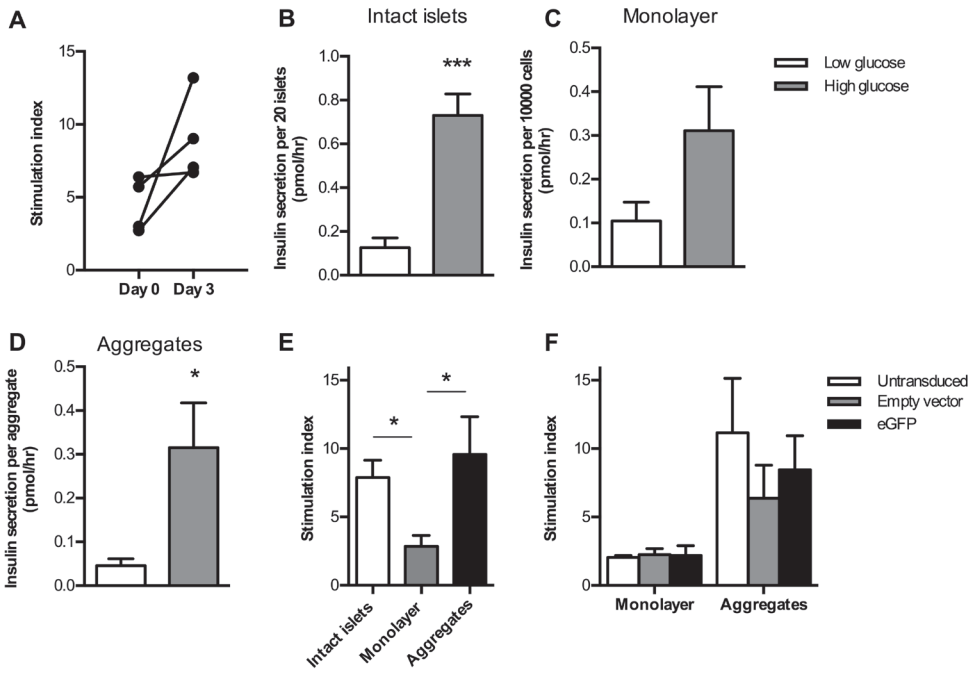

Figure 3. Glucose-induced insulin secretory response of intact islets, islet cells in monolayer and islet cell aggregates. A. Insulin release expressed as ratio of the insulin secretory response during high (16.7 mmol/l) glucose and low (2 mmol/l) glucose stimulation (stimulation index) of intact human islets after culture for 0 or 3 days in 96-well plates (n=4 donors). B-D. Insulin secretion from intact islets (B, n=8 donors), islet cells in monolayer (C, n=3 donors) and aggregated islet cells (D, n=4 donors) in response to ≥16.7 mmol/l glucose (*High glucose*). E. Stimulation index of glucose-induced insulin secretion. Intact islets: n=14 donors, islet cells in monolayer: n=5 donors, and aggregated islet cells: n=8 donors. F. Stimulation index of glucose-induced insulin secretion in monolayer (n=2 donors) and aggregated islet cells (n=3-4 donors) untransduced or transduced with an empty adenovirus vector or an adenovirus expressing shRNA against eGFP as a negative control (MOI = 2), 3 days post-transduction. *p<0.05, ***p<0.001.

In vitro exposure of human islet cells to GLP-1R agonists does not significantly enhance glucose-induced insulin secretion

The effect of glucagon-like peptide-1 receptor (GLP-1R) agonism on enhancement of glucose-induced insulin secretion was assessed for the three culture platforms. When intact human islets were exposed to glucose and different concentrations of the GLP-1R agonist exendin-4 or liraglutide, only a minor enhancement of insulin secretion by 100 nM liraglutide was observed (Fig. 4A). Furthermore, there was no significant enhancement of glucose-induced insulin secretion by GLP-1R agonist treatment in islet cells in monolayer or islet cell aggregates (Fig. 4B, Fig. 5A-C).

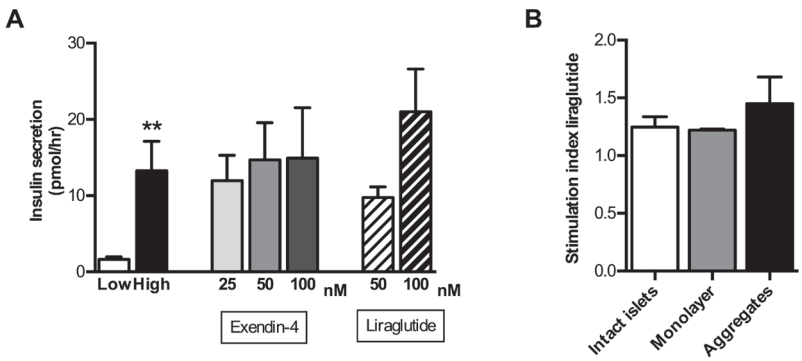

Figure 4. Insulin secretory responses of intact human islets, human islet cells in monolayer and islet cell aggregates to GLP-1R agonist treatment. A. Insulin secretion from intact islets (*n*=2-5 donors) in response to 2 mM glucose (low) and ≥16.7 mmol/l glucose (high) alone and in combination with different concentrations of exendin-4 or liraglutide. B. Stimulation index of liraglutide (100 nM)-induced enhancement of insulin secretion in intact human islets (*n*=11 donors), human islet cells in monolayer (*n*=2 donors) and islet cell aggregates (*n*=4 donors). ***p*<0.01 vs. low glucose.

Activation of cAMP/EPAC-2 signal transduction and inhibition of K-channels enhances glucose-induced insulin secretion in intact human islets and islet cell aggregates

To evaluate whether the signaling pathway activated after GLP-1 receptor activation was functional, we assessed the effect of cAMP/EPAC-2 signal transduction on insulin secretion in our culture systems. Increasing cAMP levels by forskolin and IBMX resulted in ~2.5x enhancement of glucose-induced insulin secretion compared to control in intact human islets (Fig. 5A). A similar induction of insulin secretion was reached with an EPAC-2 agonist in intact islets. However, neither forskolin and IBMX, nor EPAC-2 agonism significantly enhanced insulin secretion from islet cells in monolayer (Fig. 5B). In contrast, reaggregated islet cells showed increased glucose-induced insulin secretion upon stimulation with forksolin and IBMX or the EPAC-2 agonist (Fig. 5C). K-channel inhibition of intact human islets using TEA resulted in ~2x potentiation of insulin secretion and a similar response was observed in islet cell aggregates (Fig. 5A, C). Conversely, exposure of islet cells in monolayer to TEA did not result in enhancement of glucose-induced insulin secretion (Fig. 5B). Finally, the stimulation indices for the insulin secretagogues forskolin and IBMX, EPAC-2 agonist and TEA were reproducible in different donors and similar between intact islets and islet cell aggregates derived from the same donor (Fig. 5D-F).

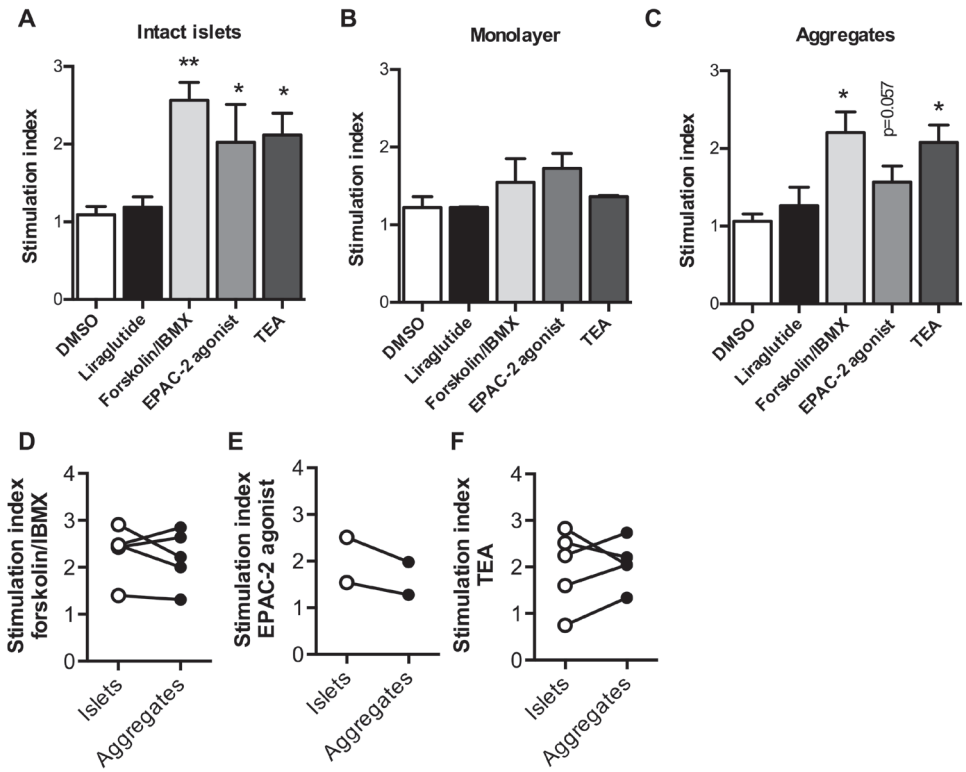

Figure 5. Insulin secretory responses to elevation of cAMP, EPAC-2 agonism and K-channel inhibition of intact islets. A-C. Stimulation index of high glucose (16.7 mM) in the presence of liraglutide (100 nM), forskolin (10 μM) and IBMX (100 μM), EPAC-2 agonist (100 μM), or TEA (100 μM) over high glucose alone in (A) intact human islets (*n*=2-7 donors), (B) human islet cells in monolayer (*n*=2 donors) and (C) islet cell aggregates (*n*=3-5 donors). D-F. Stimulation index of forskolin and IBMX (D), EPAC-2 agonist (E) and TEA (F) in intact islets or aggregates expressed per donor. **p*<0.05 and ***p*<0.01 vs. DMSO control.

The use of intact human islets, islet cells in monolayer or islet cell aggregates for high-throughput screening purposes

We evaluated the use of three different culture platforms in microwell plates using primary human islets. Table 1 summarizes the main characteristics of the three culture platforms that were compared in this study. These platforms can ultimately be used for medium to high-throughput screening of viral-delivered shRNA or small compound libraries to identify hits that can enhance insulin secretion in human islets. We therefore calculated the number of compounds that can be tested on 10,000 human islets (in quadruplicates) per set-up showing their ability to serve as a platform for medium to high-throughput screening purposes.

	Intact islets	Monolayer	Aggregates
Glucose responsiveness	yes	yes (reduced)	yes
Insulin secretagogues responsiveness	yes	no	yes
Transduction efficiency	low	high	high
No of compounds tested per 10.000 islets (quadruplicates)*	125	360	190

Table 1. Culture platform characteristics.
***Calculations were done based on the assumption that 1 islet consists of ~1.500 islet cells.

Discussion

In this study, we present a high-throughput culture platform in which the insulin secretory response of human islets is used as readout. In previous studies using rodent-derived cell lines, platforms were used in which ATP-levels were used as a proxy indicator for islet function (12), insulin protein content (6) or insulin gene expression (7). However, the biology of islets from rodents is very different from humans (13). One of the few culture systems using human islet cells is the multiparameter high-throughput screening by Hill et al. (4). However, in this platform gene expression was assessed as indicator for the ability of natural compounds to modulate insulin and Pdx1. Also, Kiselyuk et al. (5) who used a cell line derived from human islets, used insulin promoter activity as readout. Following glucose stimulation only a fraction of the total insulin content is secreted from β-cells, which makes these readouts poor indicators of secretory function. Since insulin gene expression and protein content do not directly correlate to β-cell function (8), measurement of insulin secretion is a more accurate reflection of islet function.
In this study we show that human islet cells cultured in monolayer show reduced glucose-responsiveness compared to intact islets. In the platform set up by Walpita et al. (14) human islet cells were also cultured in monolayer on ECM to screen for compounds that could enhance β-cell proliferation. The magnitude of the glucose-responsiveness of this study and our results for the monolayer culture are similar. Interestingly, in our study the insulin secretory function of human islet cell aggregates was comparable to intact human islets. This is in line with the observation that mouse MIN6 pseudoislets show improved insulin secretion compared to a monolayer culture of MIN6 cells (15). Also, Wojtusciszyn et al. (16) showed that insulin secretion from human β-cells improved when β-cells were paired with another β- or α-cell. Altogether these results show that the glucose-induced insulin secretory response of reaggregated islet cells is more in line with secretory capacity of intact human islets than of islet cells cultured in a monolayer.
From rodent studies it is known that activation of the GLP-1 receptor, which is expressed on β-cells, can potently enhance glucose-induced insulin secretion *in vitro* (17). In our study GLP-1 receptor activation did not significantly increase insulin secretion from human islet cells in any of the culture platforms. Recently, Hodson et al. (18) showed that loss of cell-cell communication results in decreased incretin-stimulated insulin secretion in human islets, which may explain

the low responsiveness in our monolayer culture. Furthermore, Hansen et al. (19) failed to see the expected increment in insulin release by exposing intact human islets to exogenous GLP-1. Also, α-cells within intact islets may already secrete GLP-1 locally, which may prevent further enhancement of insulin secretion by exogenously provided GLP-1R agonists (19, 20). Since α-cells are present in all three culture platforms, this may have interfered with the insulin secretory response of β-cells to exogenous GLP-1 analogues.

Nevertheless, direct stimulation of cAMP/EPAC-2 signal transduction, one of the main signaling pathways activated by GLP-1 receptor activation, resulted in enhancement of insulin secretion in intact islets and islet cell aggregates. This response was absent in islet cells in monolayer. In addition, closure of K-channels in β-cells resulted in increased insulin secretion from intact islets and islet cells in aggregates, but not from islet cells in monolayer. Together these results show that the aggregation of human islet cells is necessary to remain responsive to several insulin secretagogues. Whether this is also attributed to the loss of cell-cell communication as described for incretin-stimulated insulin secretion remains an open question (18). For screening platforms in which efficient virus transduction is required, reaggregation of cells into islet cell aggregates is therefore preferred over monolayer culture.

The culture platforms presented in this study show that human islets can be used for high-throughput screening assays to identify targets or compounds that enhance human islet function. Also, these systems can be applied to study other aspects of islet cell biology, such as β-cell proliferation and apoptosis. This could lead to the identification of novel mechanisms involved in β-cell function and survival.

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Chapter 8

Summary and general discussion

Diabetes mellitus affects approximately 1 million people in The Netherlands (1). Diabetes is characterized by an absolute or relative deficiency in insulin secretion from β-cells, leading to an impaired glucose homeostasis. For these patients, therapies that restore, maintain or prevent loss of functional β-cells are needed. Therefore, it is critical to understand how the β-cell mass is regulated. When the demand for insulin is chronically increased by physiological or pathological changes, the endocrine pancreas can adapt by increasing insulin secretion via an enhanced β-cell function and/or by increasing β-cell mass in order to maintain glucose homeostasis. Both obesity and pregnancy lead to insulin resistance and multiple studies have associated these conditions with an increased β-cell mass in humans (2–9). Inadequate β-cell adaptation leads to the development of hyperglycemia and eventually diabetes mellitus.

β- And α-cell adaptation are topologically heterogeneous

The pancreas is a regionally heterogeneous organ. During embryonic development the pancreas develops from two epithelial buds. The ventral bud gives rise to the posterior part of the head and the uncinate process and the dorsal bud to the anterior part of the head, body and tail of the mature pancreas (10, 11). Pancreatic islets from the ventral bud contain more cells producing pancreatic polypeptide (PP), whereas islets from the dorsal bud contain more α-cells and secrete more insulin upon glucose stimulation (12, 13). Also, several studies show that the relative area of islets in the tail-region of the pancreas is higher compared to the head and body region in humans (6, 14, 15). However, it is unknown whether β-cell adaptation to an increased insulin demand occurs homogeneously throughout the pancreas.

In **chapter 2** we describe a study in mice, which were fed a high-fat diet (HFD) to induce insulin resistance or a control diet. The pancreas was divided in a duodenal, gastric and splenic region (corresponding to the head, body and tail-region of the human pancreas, respectively) (Figure 1) and β-cell mass, β-cell proliferation and insulin secretory function of islets were studied. After 6 weeks of diet no change in β-cell mass was apparent yet, however, β-cell proliferation and glucose-induced insulin secretion were significantly higher in islets derived from the splenic region compared to islets derived from the duodenal and gastric region of the pancreas (Table 1). We therefore conclude that β-cell adaptation is topologically heterogeneous in response to HFD in mice. Also, α-cell mass was found to be decreased in the splenic region only after 6 weeks HFD (data not shown).

Next, we assessed whether β-cell adaptation is topologically heterogeneous in a different animal model of insulin resistance. Glucocorticoid-induced insulin resistance occurs within 5 days of treatment (16) and is therefore an acute stimulus for β-cell adaptation. We investigated β-cell adaptation throughout the pancreas in glucocorticoid-induced insulin resistance in rats (**chapter 3**). After 6 weeks, the β-cell area was significantly increased in DXM-treated rats, and this increase

mainly occurred in the splenic region of the pancreas. This increase was associated with an enlarged β-cell cluster size while no change in β-cell proliferation was observed after 3 and 6 weeks of treatment.

Subsequently, we wondered whether β-cell mass adaptation in humans would be topologically heterogeneous as well. **Chapter 4** describes a study in which we examined the β-cell mass and glucagon-producing α-cell mass of 15 non-diabetic obese and 15 lean age-matched human subjects in the head (excluding regions that were rich in PP cells), body and tail region. Both β- and α-cell area were the highest in the tail-region of the pancreas (Table 1). In obese subjects β- and α-cell mass were increased and both β- and α-cell area were significantly higher in the head-region of the pancreas compared to lean controls, whereas islet density was significantly increased in the tail-region. The α- to β-cell ratio was similar throughout the pancreas and preserved following adaptation in non-diabetic obese subjects. Altogether these data show that in obese human subjects β- and α-cell mass adaptation is topologically heterogeneous.

	Model	Head / DR	Body / GR	Tail / SR
Insulin secretory function	Mice	=	=	↑
β-Cell mass	Mice	=	=	↑
	Rats*	=	=	↑
	Humans*	↑	=	=
α-Cell mass	Mice	=	=	↓
	Humans*	↑	=	=

Table 1. Changes in insulin secretory function, β-cell area and α-cell area of different pancreatic regions compared in HFD-fed vs. control mice (Mice), DXM-treated vs. control rats (Rats) and obese vs. lean human subjects (Humans). *β-cell area. DR = duodenal region, GR = gastric region, SR = splenic region.

Human versus rodents

In this thesis we show for the first time that β-cell adaptation to an increased insulin demand is topologically heterogeneous throughout the pancreas of mice, rats and humans (Table 1, **chapters 2 - 4**). In rodents, islets derived from the splenic region of the pancreas are involved in the first line of response in β- and α-cell adaptation. In obese humans the islet density was mostly increased in the tail-region of the pancreas. Nevertheless, the most prominent increase in β-cell area was observed in the head-region of the pancreas of obese human donors; which also appeared to be the region showing a preferential loss of β-cells in patients with type 2 diabetes (17). Dissimilarities observed between the study results from humans versus rodents can obviously be attributed to differences in species. Several differences between rodents and human endocrine pancreas have been observed (18). The gross morphology of the mature pancreas is different between rodents and humans (Fig. 1A, B). The rodent pancreas has a lobular structure of loosely connected tissue that aligns the spleen, stomach and upper part of the intestine whereas the human pancreas is a single, compact organ surrounded by a fibrous stroma. In rodents, the

majority of the islets consists of β-cells that are surrounded by a single layer of α-cells, whereas in humans endocrine cells are more mixed throughout the islets resulting in more heterologous contacts between α- and β-cells (Fig. 1 C, D) (19, 20). Also, the proportion of β-cells in humans islets is on average 55% versus 77% in mice, whereas about 38% of the human islet is composed of α-cells compared to 18% of the mouse islet (19). In humans, we show that the β-cell area is the highest in the tail-region of the pancreas (**chapter 4**); which is in line with previous observations in human donor pancreases (6, 14, 15). However, the β-cell area was similar throughout the pancreas of mice fed a control diet for 12 weeks (**chapter 2**). Hornblad et al. (21) reported that the β-cell area was the highest in the head-region of the pancreas of 8 weeks old mice. Together these studies indicate that the head-region of the pancreas in humans may not necessarily correlate to the head-region of the mouse pancreas.

Furthermore, in the splenic region of the pancreas in HFD-fed mice we observed an increase in β-cell proliferation (BrdU labeling for 7 days), whereas β-cell proliferation (identified by Ki67 as a proliferation marker) was not changed in DXM-treated rats after 3 and 6 weeks of treatment. Previous studies have observed increased β-cell proliferation after 3 days of DXM-treatment (22–24), suggesting that the peak in β-cell proliferation induced by DXM-treatment in our study occurred within the first 3 weeks. β-Cell proliferation was rarely observed in the human pancreas donors and was not different between regions. It should be noted that the animals in our studies (mice and rats were ~8 weeks old at the start of the study) could be considered young adults, whereas the average age in our study of human pancreas donors was approximately 50 years. As it is well known that β-cell proliferation and adaptation are negatively affected by ageing (25, 26), this may have contribute to observed differences as well.

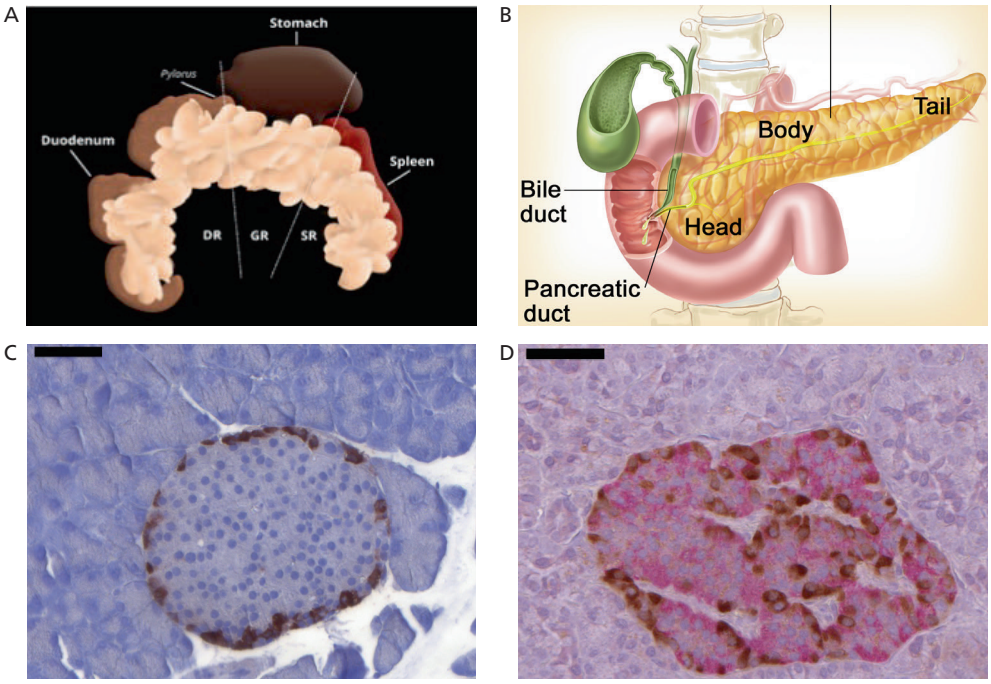

Figure 1. Mouse versus human pancreas and islets. A. Gross anatomy of mouse pancreas, DR = duodenal region, GR = gastric region, SR = splenic region. B. Gross anatomy of human pancreas. *Reprinted with permission from Terese Winslow*. C. Representative image of a mouse islet, with α-cells (brown) surrounding β-cells (center of the islet). Scale bar = 50 μm. D. Representative image of a human islet, α-cells (brown) are intermingled with β–cells (red). Scale bar = 50 μm.

Mechanisms and stimuli of topologically heterogeneous β-cell adaptation

The observed regional heterogeneity in β-cell adaptation in response to a HFD stimulus, DXM-treatment or in obese humans could be explained in two ways: the islets from different pancreatic regions are intrinsically different or, the islets receive distinct extrinsic signals from their microenvironment within the pancreas. In **chapter 2** we assessed this latter hypothesis, by transplantation of untreated mouse islets from the three pancreatic regions to an extrapancreatic location in diabetic mice, in which the increased demand for insulin will stimulate β-cell regeneration. After 10 days, no difference between islets isolated from different regions was found. These results suggest that the observed topological heterogeneity of β cell adaptation in HFD-fed mice is most likely the result of distinct extrinsic signals present in the microenvironment of the islet within the pancreas.

Stimuli that have been identified to affect β-cell proliferation comprise several growth factors and hormones. These proteins are often produced by other organs than the pancreas, such as the liver, adipose tissue and the intestine, and released in the vasculature. Islets are highly vascularized to enable efficient secretion of insulin and glucagon into the circulation; they receive

per unit weight about 20 times more arterial blood compared to the exocrine pancreas in rats (27, 28). Differences in vascular density between pancreatic regions could result in heterogeneous exposure to growth factors and hormones. By using *in vivo* labeling methods, Lau et al. (29) characterized a subpopulation of mouse islets (5%) with a greater blood perfusion and vascular density that were associated with increased β-cell function and proliferation. Recently, intravital blood vessel labeling revealed that the islet vascular supply increases during insulin resistance by dilation of preexisting vessels in mice (30). In addition, this same study showed that islets of insulin resistant mice have increased global islet innervation visualized by the labeling of the neuronal marker neuronal class II β-tubulin (TUJ1). Islets are densely innervated by the autonomic nervous system (31) and it was reported that obesity-induced β-cell mass expansion is regulated through neuronal signals from the liver (32).

It remains unclear whether changes in vascular supply or innervation are the cause or consequence of β-cell mass adaptation. Islet cells produce angiogenic factors including vascular endothelial growth factor (VEGF)-A, which is one of the principal regulators of vascular homeostasis (33). Interestingly, VEGF-A expression was increased in the subpopulation of islets having a greater blood perfusion (29). Also, it has been reported that α-cells of human islets provide cholinergic signals to neighboring β-cells, thereby priming β-cell function in a paracrine way (34).

Furthermore, islets are structurally and functionally closely related to the exocrine pancreas. This is referred to as the islet-acinar axis, in which exocrine functions are regulated by insulin and somatostatin (35). The content of one of the main enzymes produced by acinar cells, amylase, was found to be higher in the dorsal region compared to the ventral region of the pancreas in rats (36). Also, in the field of regenerative studies there appears to be a strong link between exocrine acinar cells and β-cells, which share their endodermal origin. One of the first studies showing that adult cells can be reprogrammed into another adult cell type, without reversion to a pluripotent stem cell state, showed the conversion of adult exocrine cells to β-cells by expressing three transcription factors (*Ngn3*, *Pdx1* and *MafA*) in mice (37). Recently it was shown that the β-cell mass of alloxan-induced diabetic mice was regenerated by acinar-to-β-cell reprogramming, without genetic modification, through transient cytokine exposure (38). In addition to demonstrating a potential source for de novo β-cell generation, these studies illustrate the intimate relation between islets and its exocrine environment. Future research should clarify whether the exocrine tissue surrounding islets is involved in the regulation of β-cell adaptation.

Implications for future research

Most importantly, the results in **chapters 2 - 4** imply that quantification of the β- and α-cell mass in animal or human pancreases should be based on representative samples throughout the entire organ. In most histological studies of α- and/or β-cell adaptation the head-region of the human pancreas was not included (5–7, 39, 40), which may have led to an incorrect estimation of actual changes in these studies. Furthermore, comparison of regional differences in β-cell adaptation may lead to the identification of novel factors involved in β-cell mass growth and function.

β-Cell adaptation in response to different metabolic stimuli

In this thesis we studied β-cell adaptation in response to different metabolic changes (Table 2). One of the main stimuli for β-cell adaptation is insulin resistance. In **chapters 2 – 4** we show that insulin resistance is associated with an increased β-cell mass in different species. High-fat diet induced insulin resistance in mice led to an increased β-cell function, β-cell proliferation and β-cell mass as a compensatory response to the increased demand for insulin (**chapter 2**). Also in human obesity, which is associated with insulin resistance, an increased β-cell mass was observed (**chapter 4**). In **chapter 3** we show that DXM treatment results in an increased insulin secretory response after a glucose load that is associated with an increased β-cell mass after 3 weeks.

One of the most potent hormones that can enhance both β-cell function and β-cell proliferation is the incretin glucagon-like peptide-1 (GLP-1). In animal models of diabetes, GLP-1 receptor agonist (GLP-1RA) treatment increases the β-cell mass (41–43). GLP-1 based therapies improve glycemic control in patients with type 2 diabetes and are associated with reduced blood pressure, improved lipid profiles and improved endothelial function (44, 45). Therefore, these compounds have also been evaluated in non-diabetic individuals with obesity and cardiovascular disease (46–49). However, their effect on β-cell mass in these normoglycemic conditions, in which there is no increased demand for insulin, is not clear. In **chapter 5** we studied the effects of the GLP-1RA liraglutide on β-cell mass and function in normoglycemic mice. Mice were treated with liraglutide or PBS and fed a control or HFD for 1 or 6 weeks. Treatment with liraglutide for 6 weeks led to increased insulin sensitivity and attenuation of HFD-induced insulin resistance. After 6 weeks of treatment a reduction in β-cell mass was observed in liraglutide-treated control and HFD-fed mice. This was associated with a lower β-cell proliferation rate after 1 week of treatment. Islets isolated from liraglutide-treated control mice showed an enhancement of glucose-induced insulin secretion. Together these data show that GLP-1RA treatment in normoglycemic mice leads to increases in insulin sensitivity and β-cell function that are associated with a reduction in β-cell mass in order to maintain normoglycemia.

Nutrients like glucose and free fatty acids can modulate β-cell mass growth and function (50–54). In many popular weight loss diets the amount of fat is substantially increased at the expense of carbohydrates. Such diets force the body to use fats instead of carbohydrates as primary source of energy. However, the long-term effects of these high-fat low-carbohydrate ketogenic diets (KD) on pancreatic endocrine cells are unknown. We hypothesized that a long-term KD creates a metabolic environment in which there is a decreased demand for insulin and an increased demand for glucagon to stimulate gluconeogenesis. **Chapter 6** describes a study in which mice were fed a KD for 22 weeks. Despite an initial weight loss, KD did not result in weight loss after 22 weeks. Long-term KD resulted in glucose intolerance that was associated with insufficient insulin secretion from β-cells. After 22 weeks, the β-cell mass was found to be reduced in KD-fed mice compared to controls. Together our data show that long-term KD causes dyslipidemia,

a proinflammatory state, signs of hepatic steatosis, glucose intolerance, and a reduction in β-cell mass, but no weight loss. This indicates that long-term KD leads to features that are also associated with the metabolic syndrome and an increased risk for type 2 diabetes in humans.

Model	Species	Insulin secretory function	β-Cell mass
Obesity	Humans		↑
DXM	Rats	↑	↑
HFD	Mice	↑	↑
GLP-1RA	Mice	↑	↓
HFD + GLP-1RA	Mice	↑	↓
KD	Mice	↓	↓

Table 2. Changes in insulin secretory function and β-cell mass in obese vs. lean human subjects (Obesity), DXM-treated vs. control rats (DMX), HFD-fed vs. control mice (HFD), liraglutide-treated vs. control mice (GLP-1RA), liraglutide-treated HFD-fed vs. control mice (HFD + GLP-1RA), ketogenic diet-fed vs. control mice (KD). DXM = dexamethasone, HFD = high-fat diet, GLP-1RA = glucagon-like peptide 1 receptor agonist, KD = ketogenic diet.

Mechanisms of β-cell adaptation in response to different metabolic stimuli

GLP-1RA can lead to different effects on insulin secretion and β-cell proliferation under normoglycemic conditions

The effect of GLP-1-based therapies on insulin secretion from β-cells has been reported to be glucose-dependent (55). No insulin secretory response was observed from isolated perfused rat pancreas to GLP-1 stimulation at a glucose concentration of 2.8 mM, whereas insulin secretion was increased when glucose concentrations were raised to 6.6 and 16.7 mM (51). We show in **chapter 5** that sustained GLP-1RA treatment during normoglycemic conditions is associated with increased insulin secretion from isolated islets, in the absence of direct GLP-1RA stimulation. These results imply that GLP-1RA treatment during normoglycemic conditions enhances insulin secretion. In contrast, GLP-1RA treatment during normoglycemic conditions did not enhance β-cell mass in mice. Moreover, the increased insulin sensitivity and enhancement of insulin secretion resulted in a decreased need for new β-cells, resulting in a decrease in β-cell proliferation and a reduced β-cell mass in GLP-1RA treated normoglycemic mice. This is in line with the observation by Porat et al. that glucose-driven glycolysis is one of the key drivers for β-cell proliferation (56).
In our study, GLP-1RA treatment during normoglycemia resulted in increased insulin secretion whereas β-cell proliferation was reduced. Activation of the GLP-1R on β-cells leads to an increase in cyclic AMP (cAMP) concentrations, which is a key messenger in β-cells (Fig. 2) (55, 57). Activation of its signaling pathways has been reported to stimulate insulin secretion and β-cell proliferation (58). It is unknown how GLP-1RA treatment regulates these two different effects in

the β-cell. cAMP signals are transduced via two pathways in the β-cells, the cAMP-dependent protein kinase A (PKA) and the exchange protein activated by cAMP (EPAC). Both PKA and EPAC have been implicated in transducing the beneficial effects on β-cell function and the protection of β-cell mass (58, 59). Recent studies have shown the predominant role for PKA-dependent signaling for β-cell function *in vivo* (60, 61). PKA activity is transduced either to transcriptional events through PKA phosphorylation of the transcription factor cAMP response element-binding protein or by the formation of complexes with A-kinase anchoring proteins (AKAPs). AKAPs are a family of intracellular-signaling adaptor proteins that direct PKA to locations within the cell where it can exert specific effects (57, 62). Future research should elucidate which AKAP complexes are involved in regulation of insulin secretion or β-cell proliferation to understand the mechanisms by which cAMP/PKA signaling is regulating both β-cell function and mass.

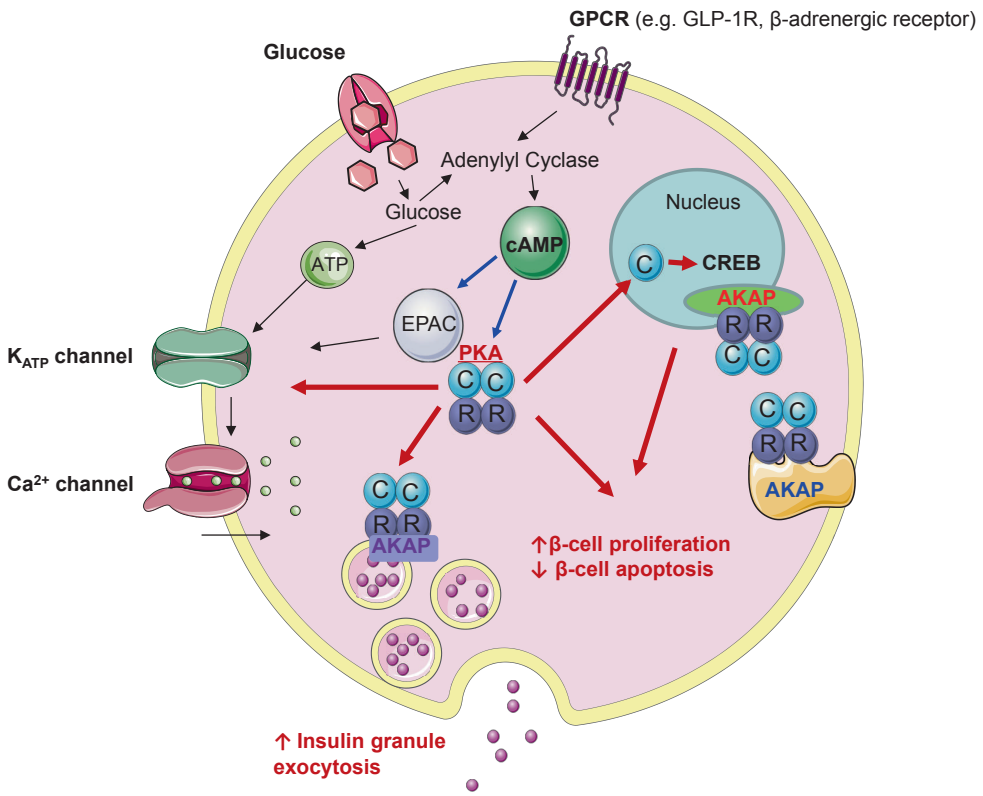

Figure 2. Schematic overview of cAMP/PKA signaling pathway in the β-cell. Red arrows indicate the potential different pathways by which cAMP regulates β-cell function and survival. GPCR=G-protein coupled receptor; GLP-1R=Glucagon-like peptide-1 receptor; cAMP=cyclic AMP; PKA=cAMP-dependent protein kinase A; EPAC=exchange protein activated by cAMP; CREB=cAMP response element-binding protein; AKAP=A-kinase anchoring proteins; R=regulatory subunit; C=catalytic subunit.

Long-term KD leads to a reduced β-cell mass and an insulin secretory defect

In **chapter 6** we show that a long-term ketogenic diet results in glucose intolerance most likely because of β-cell dysfunction and a reduction in β-cell number that result in inadequate insulin secretion. Already after 5 weeks of KD diet, mice show the first signs of glucose intolerance. The insulin secretory response is not increased to compensate for this increased demand for insulin. Ultimately insulin secretory function and the number of β-cells in mice fed a KD was reduced. This strongly suggests that the β-cell adaptive response and secretory function have become dysfunctional as a result of the long-term KD feeding. Similar to patients with type 2 diabetes (63), the reduction in β-cell mass was most prominent in the DR of the pancreas (data not shown). Long-term KD results in dyslipidemia, which can lead to β-cell dysfunction due to lipotoxicity. Chronic exposure of β-cells to increased concentrations of free fatty acids (FFA), reduces insulin secretion and induces β-cell apoptosis (64, 65). Excess FFAs can promote the expression of pro-inflammatory factors in islets, such as Il-1β (66). Il-1β, which was increased after long-term KD in our study, is a master regulator of inflammation and can inhibit insulin secretion and stimulate β-cell death (66, 67). Altogether, long-term KD leads to dyslipidemia and a pro-inflammatory state, which are associated with an impaired adaptive response of β-cell function and mass to KD-induced changes in glucose metabolism.

α-cell adaptation in response to different metabolic stimuli

Adaptation of α-cell mass in human obesity (**chapter 4**) and in normoglycemic mice receiving incretin therapy (**chapter 5**) was similar to changes in the β-cell mass and resulted in maintenance of the α- to β-cell ratio (Table 3). It has been reported that human islets prefer heterologous contacts between β- and α-cells (20). Also, insulin secretion from individual human β-cells is enhanced when they are coupled to an α-cell (68). This functional connection between β- and α-cells may explain the maintenance of the α- to β-cell ratio following adaptation to metabolic stimuli.

In contrast, the α- to β-cell ratio in long-term KD mice was decreased due to the considerable reduction of α-cell mass. This change can be a direct consequence of KD or a response to counteract glucose intolerance. During ketosis, glucagon stimulates hepatic glucose production and lipolysis to generate energy. In **chapter 6** we show that long-term KD results in a reduced insulin-stimulated glucose uptake. This could result in a negative feedback to glucagon-producing α-cells resulting in less gluconeogenesis and no further worsening of the blood glucose concentration. This is supported by the observation that circulating glucagon concentrations were decreased after 5 weeks of KD in mice (69). In order to maintain glucose homeostasis, the rate of glucose entering the circulation should be balanced by the removal of glucose out of the circulation. In this process both insulin and glucagon play a major role. Past research has shown that the insulin producing β-cell mass can adapt to changing metabolic demands (reviewed in **chapter 1**). Little

is known about the involvement of the α-cell mass in this process. In **chapters 4 - 6** we show that, in addition to adaptation of the β-cell mass, metabolic changes also affect the α-cell mass. Interestingly, in **chapter 5** we show changes in α-cell mass preceding adaptation of the β-cell mass in HFD-fed mice, which is in line previous observations in mice and non-human primates (70, 71). An imbalance between glucagon and insulin characterizes both type 1 and type 2 diabetes (72, 73). This suggests that failure of both β- and α-cell adaptation can contribute to the development of diabetes. Future research on β-cell adaptation should therefore also study changes in α-cell mass and function.

Model	Species	α-cell mass	Ratio α- to β-cells
Obesity	Humans	↑	=
HFD	Mice	↓	=
GLP-1RA	Mice	↓	=
HFD + GLP-1RA	Mice	↓	=
KD	Mice	↓	↓

Table 3. Changes in α-cell mass and the ratio α- to β-cells in obese vs. lean human subjects (Obesity), HFD-fed vs. control mice (HFD), liraglutide-treated vs. control mice (GLP-1RA), liraglutide-treated HFD-fed vs. control mice (HFD + GLP-1RA), ketogenic diet-fed vs. control mice (KD). HFD = high-fat diet, GLP-1RA = glucagon-like peptide 1 receptor agonist, KD = ketogenic diet.

Mechanistic studies of human islet adaptation

For studying β- and α-cell adaption in human islets, we currently depend on histological analyses of biopsies taken at autopsy or after pancreatectomy generating a static picture. Animal models can provide more mechanistic insight because β- and α-cell adaptation in response to metabolic changes can be studied in a controlled setting at different time points. In addition, *in vitro* biotechnology platforms can be a powerful tool to assess the influence of different metabolic stimuli and factors on human islet function and survival in order to identify new mechanisms involved in β- and α-cell adaptation. Since no assay platforms for human islet adaptation studies were available, we developed three high-throughput culture platforms for primary human islets to assess β-cell function in **chapter 7**: intact human islets, and cells from dispersed human islets cultured either in monolayer on extracellular matrix coated plates or reaggregated into islet-cell clusters. Dispersed islet cells can be efficiently transduced using adeno- and lentivirus. Activation of cAMP/EPAC-2 signal transduction and inhibition of K-channels enhanced glucose-induced insulin secretion in intact islets and islet cell aggregates, but not in monolayer culture. This shows that human islet cells behave most similar to intact human islets when cells are clustered three-dimensionally. Furthermore, these systems can also be used to study other aspects of human islet adaptation, such as α-cell function, and β- or α-cell proliferation and survival. These three culture platforms can be used in future studies for the screening of viral shRNA or small compound libraries to identify new mechanisms involved in β- and α-cell adaption of human islets.

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Chapter 9

Nederlandse samenvatting

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Nederlandse samenvatting

In Nederland lijden ongeveer 1 miljoen mensen aan diabetes mellitus. Diabetes wordt gekarakteriseerd door een absolute of relatieve tekortkoming aan de hoeveelheid insuline die de β-cellen uitscheiden. Deze β-cellen bevinden zich in de eilandjes van Langerhans in het pancreas. Het insulinetekort leidt tot een verstoorde balans in de glucosehuishouding. Voor mensen met diabetes zijn therapieën nodig die de β-celfunctie herstellen, onderhouden en het verlies van deze van deze cellen tegengaan. Het is daarom van groot belang om te begrijpen hoe het aantal β-cellen, de β-celmassa, wordt gereguleerd. Wanneer de vraag naar insuline chronisch is verhoogd door fysiologische of pathologische metabole veranderingen, zoals bijvoorbeeld gedurende een zwangerschap of bij obesitas, kan het endocriene pancreas zich aanpassen door de insulinesecretie te laten toenemen. Dit kan gebeuren via een verhoogde β-celfunctie of een vermeerdering van het aantal β-cellen. Door deze adaptatie van de β-cellen kan de balans in de glucosehuishouding behouden worden. Onvoldoende β-celadaptatie leidt tot de ontwikkeling van een verhoogde bloedsuiker en uiteindelijk diabetes mellitus.

β- en α-celadaptatie zijn topologisch heterogeen

Het pancreas is een regionaal heterogeen orgaan. Tijdens de embryonale ontwikkeling wordt het pancreas gevormd door twee epitheliale uitstulpingen. De ventrale uitstulping vormt het posteriore gedeelte van de kop en het uncinate proces, en de dorsale uitstulping vormt de kop, romp en staart van het volgroeide pancreas. Eilandjes van Langerhans die ontstaan in het ventrale gedeelte van het pancreas bevatten meer cellen die pancreas polypeptide maken, terwijl eilandjes van de dorsale uitstulping meer glucagon-producerende α-cellen bevatten. Ook scheiden deze dorsale eilandjes meer insuline uit als ze gestimuleerd worden met glucose. Daarnaast hebben diverse studies laten zien dat in het pancreas van mensen meer eilandjes in de staartregio zitten vergeleken met de kop- en rompregio. Echter, het is niet bekend of de adaptatie van β-cellen aan een toegenomen vraag naar insuline homogeen plaatsvindt door het hele pancreas.

In **hoofdstuk 2** beschrijven we een studie waarin muizen een hoog-vet dieet (HVD) gevoerd kregen, om daarmee insulineresistentie te veroorzaken, of een controledieet. We verdeelden het pancreas in drie regio's: een duodenale, gastrische en een splenische regio (respectievelijk corresponderend aan de kop-, romp- en staartregio van het pancreas in mensen). In elk gedeelte bepaalden we de β-celmassa, β-celproliferatie en β-celfunctie. Na 6 weken dieet was het aantal delende β-cellen en de β-celfunctie significant toegenomen in eilandjes afkomstig van de splenische regio van het pancreas in vergelijking met eilandjes van de duodenale en gastrische regio. Hieruit concluderen we dat de adaptatie van β-cellen in reactie op een HVD in muizen regionaal verschillend is. Ook zagen we dat de α-celmassa was afgenomen in de splenische regio van het pancreas, maar niet in de andere regio's.

Daarna hebben we onderzocht of β-celadaptatie ook topologisch heterogeen is in een ander diermodel van insulineresistentie. Glucocorticoid-geïnduceerde insulineresistentie openbaart zich binnen 5 dagen na de start van de behandeling in ratten en is daarom een acute stimulans voor β-celadaptatie. In **hoofdstuk 3** bestuderen we regionale β-celadaptatie in het pancreas van glucocorticoid-geïnduceerde insulineresistente ratten. De relatieve β-celmassa was significant toegenomen na 6 weken dexamethason (DXM) behandeling in ratten en de toename was het grootst in de splenische regio van het pancreas.
Vervolgens wilden we weten of β-celadaptatie in mensen ook topologisch heterogeen verloopt. In **hoofdstuk 4** beschrijven we een studie waarin we de β-celmassa en de glucagon-producerende α-celmassa onderzoeken in verschillende regio's van het pancreas in mensen. Hiervoor bestudeerden we het donorpancreas van 15 obese individuen zonder diabetes en 15 slanke controles met een vergelijkbare leeftijd. Zowel de relatieve β- als α-celmassa was het grootste in de staartregio van het pancreas. In obese individuen waren de β- en α-celmassa groter. In de kop-regio van het pancreas waren de relatieve β- en α-celmassa significant groter vergeleken met de slanke controles. De eilandjesdichtheid was significant hoger in de staartregio van obese individuen. Deze resultaten laten zien dat in obese individuen de β- en α-celmassa adaptatie regionaal heterogeen zijn.

	Model	Kop / DR	Romp / GR	Staart / SR
β-celfunctie	Muizen	=	=	↑
β-celmassa	Muizen	=	=	↑
	Ratten*	=	=	↑
	Mensen*	↑	=	=
α-celmassa	Muizen	=	=	↓
	Mensen*	↑	=	=

Tabel 1. Veranderingen in β–celfunctie, en de β- en α-celmassa in verschillende regio's van het pancreas in hoog-vet dieet gevoede vs. controle muizen (Muizen), dexamethason-behandelde vs. controle ratten (Ratten) en obese vs. slanke mensen (Mensen). *relatieve celmassa: afgeleid van gemeten cel- en pancreasoppervlaktes. DR = duodenale regio, GR = gastrische regio, SR = splenische regio.

De heterogeniteit in adaptatie van β-cellen uit verschillende regio's van het pancreas in reactie op een verhoogde vraag naar insuline kan op twee manieren verklaard worden: (i) de eilanden afkomstig van de verschillende pancreas regio's zijn intrinsiek verschillend, of, (ii) de eilanden in elk van de regio's in het pancreas ontvangen andere extrinsieke signalen via bv. de bloedtoevoer, zenuwimpulsen of het omliggende exocriene weefsel van het pancreas. In **hoofdstuk 2** testen we deze laatste hypothese door eilandjes afkomstig uit verschillende pancreasregio's van onbehandelde muizen te transplanteren naar een locatie buiten het pancreas (onder het nierkapsel) in muizen met diabetes. In een muis met diabetes is de vraag naar insuline sterk verhoogd. Dit resulteert in aanpassing van de getransplanteerde eilandjes om daarmee de hoeveelheid insuline te verhogen. Tien dagen na de transplantatie hadden eilandjes afkomstig uit

de verschillende regio's van het pancreas zich op dezelfde manier aangepast. Dit suggereert dat de heterogeniteit in adaptatie van eilandjes die we eerder in HVD-gevoede muizen observeerden het resultaat is van extrinsieke signalen die aanwezig zijn in het micromilieu van de eilandjes in het pancreas.
De resultaten uit **hoofdstuk 2 – 4** impliceren dat kwantificatie van de β- en α-celmassa in het pancreas van dieren en mensen gebaseerd moet zijn op representatieve monsters uit het gehele orgaan. In de literatuur worden histologische studies naar de β- en/of α-celmassa beschreven waarbij de kopregio van het pancreas vaak niet is meegenomen. Dit kan in deze studies geleid hebben tot een incorrecte inschatting van de veranderingen in de endocriene celmassa. Daarnaast kan het vergelijken van regionale verschillen in β-celadaptatie leiden tot de identificatie van nieuwe factoren die betrokken zijn bij de groei en functie van β-cellen.

β-celadaptatie in reactie op diverse metabole stimuli
In dit proefschrift bestuderen we de adaptatie van β-cellen in reactie op verschillende metabole veranderingen (tabel 2). Een van de belangrijkste stimuli voor adaptatie van β-cellen is insulineresistentie. In de **hoofdstukken 2 - 4** laten we zien dat insulineresistentie leidt tot een vergrote β-celmassa in knaagdieren en mensen. HVD-geïnduceerde insulineresistentie in muizen leidt tot een toename van de β-celfunctie, β-celproliferatie en β-celmassa ter compensatie voor de toegenomen vraag naar insuline (**hoofdstuk 2**). Ook obesitas bij mensen, dat vaak samen gaat met insulineresistentie, is geassocieerd met een grotere β-celmassa vergeleken met slanke individuen (**hoofdstuk 4**). In **hoofdstuk 3** zien we dat DXM behandeling van ratten gedurende 3 weken resulteert in een verhoging van de insulinesecretierespons na een glucosestimulus en een toename van de β-celmassa.
Een van de meest potente hormonen die zowel β-celfunctie als β-celproliferatie kan stimuleren is de incretine glucagon-like peptide-1 (GLP-1). In diermodellen van diabetes is aangetoond dat behandeling met GLP-1 receptor agonisten (GLP-1RA) de β-celmassa kan vergroten. Therapieën gebaseerd op het werkingsmechanisme van GLP-1 verbeteren de glycemische controle in patiënten met type 2 diabetes. Vanwege positieve effecten op lichaamsgewicht en lipidewaarden in het bloed worden deze therapieën nu ook geëvalueerd in individuen met obesitas en/of hart- en vaatziekten die geen diabetes hebben. De gevolgen van deze behandeling op de β-celmassa tijdens normoglycemische condities, waarbij er geen vraag is naar extra insuline, is echter niet duidelijk. In **hoofdstuk 5** bestuderen we het effect van de GLP-1RA liraglutide op de β-celmassa en β-celfunctie in normoglycemische muizen. Muizen werden met liraglutide of PBS behandeld en kregen een controle of een HVD gedurende 1 of 6 weken. Zes weken behandeling met liraglutide resulteerde in een toegenomen gevoeligheid voor insuline en voorkwam HVD-geïnduceerde insulineresistentie. Na 1 week behandeling met liraglutide was het aantal delende β-cellen sterk verminderd wat resulteerde in een kleinere β-celmassa na 6 weken behandeling. Geïsoleerde eilanden van met liraglutide behandelde normoglycemische muizen lieten een

toename zien van glucose-geïnduceerde insulinesecretie. Deze data laten zien dat GLP-1RA behandeling van normoglycemische muizen kan leiden tot toename van de gevoeligheid voor insuline en β-celfunctie. Dit is geassocieerd met een reductie van de β-celmassa om daarmee de glucosehuishouding in balans te houden.

Voedingsstoffen zoals glucose en vrije vetzuren kunnen de groei en functie van β-cellen beïnvloeden. In een aantal populaire gewichtsverminderende diëten is de hoeveelheid vetten substantieel verhoogd ten koste van koolhydraten. Dergelijke diëten dwingen het lichaam om als primaire energiebron vetten in plaats van koolhydraten te gebruiken. De langetermijneffecten van deze hoog-vet laag-koolhydraat ketogene diëten op endocriene cellen in het pancreas zijn echter onbekend. Een langdurig ketogeen dieet zou een metabole omgeving kunnen creëren waarin de vraag naar insuline sterk is verlaagd, terwijl de vraag naar glucagon verhoogd zou kunnen zijn om hiermee gluconeogenese te stimuleren. **Hoofdstuk 6** beschrijft een studie waarin muizen gedurende 22 weken een ketogeen dieet gevoed kregen. Hoewel er in de eerste weken van het dieet een gewichtsverlies optrad, resulteerde het ketogeen dieet na 22 weken niet in een verminderd gewicht. Een langdurig ketogeen dieet leidde tot glucose-intolerantie, dat geassocieerd was met een tekort aan insulinesecretie door de β-cellen. Na 22 weken was de β-celmassa kleiner in ketogeen dieet-gevoede muizen vergeleken met controlemuizen. Onze data laten zien dat een langdurig ketogeen dieet leidt tot dyslipidemia, een proinflammatoire staat, tekenen van leversteatose, glucose-intolerantie en afname van de β-celmassa, maar geen gewichtsverlies. Deze resultaten suggereren dat een langdurig ketogeen dieet leidt tot symptomen die ook zijn geassocieerd met het metabole syndroom en een toename op het risico voor type 2 diabetes bij mensen.

Model	Soort	β-celfunctie	β-celmassa
Obesitas	Mensen		↑
DXM	Ratten	↑	↑
HVD	Muizen	↑	↑
GLP-1RA	Muizen	↑	↓
HVD + GLP-1RA	Muizen	↑	↓
KD	Muizen	↓	↓

Tabel 2. Veranderingen in β–celfunctie en β-celmassa in obese vs. slanke mensen (Obesitas), dexamethason-behandelde vs. controle ratten (Ratten), HVD-gevoede vs. controle muizen (HVD), liraglutide-behandelde vs. controle muizen (GLP-1RA), liraglutide-behandelde HVD-gevoede vs. controle muizen (HVD + GLP-1RA), KD dieet-gevoede vs. controle muizen (KD). DXM = dexamethason, HVD = hoog-vet dieet, GLP-1RA = glucagon-like peptide 1 receptor agonist, KD = ketogeen dieet.

α-celadaptatie in reactie op diverse metabole stimuli

Voor de glucosehomeostase is het van belang dat de hoeveelheid glucose die de circulatie binnenkomt en uitgaat in balans is. In dit proces spelen zowel insuline als glucagon een belangrijke rol. Eerder onderzoek heeft aangetoond dat de insulineproducerende β-celmassa zich

kan aanpassen aan veranderde metabole omstandigheden. Er is echter weinig bekend over de rol van de α-celmassa in dit proces. In **hoofdstuk 4 – 6** laten we zien dat metabole veranderingen tot aanpassing van zowel de β- als de α-celmassa leiden.

De aanpassing van de α-celmassa in obese mensen (**hoofdstuk 4**) en in normoglycemische muizen onder behandeling van de GLP-1RA liraglutide (**hoofdstuk 5**) was vergelijkbaar met de veranderingen die optraden in de β-celmassa, en resulteerde in het behoud van de balans tussen α- en β-cellen (tabel 3). Uit literatuur weten we dat in humane eilanden er veel celcontacten zijn tussen α- en β-cellen en dat dit contact resulteert in een verhoging van de insulinesecretie. Deze functionele verbinding tussen α- en β-cellen zou kunnen verklaren waarom de verhouding tussen α- en β-cellen behouden blijft in reactie op metabole veranderingen. In **hoofdstuk 5** zien we dat veranderingen in de α-celmassa plaatsvinden voordat deze in de β-celmassa zichtbaar worden in HVD-gevoede muizen. In **hoofdstuk 6** laten we zien dat de ratio α- tot β-cellen in muizen die langdurig een ketogeen dieet ontvingen was verlaagd door een sterke reductie in de α-celmassa. Deze verandering kan een directe consequentie zijn van het ketogeen dieet of een aanpassingsreactie van de α-celmassa om zo de ontstane glucose-intolerantie tegen te gaan.

Een disbalans tussen glucagon en insuline is karakteristiek voor zowel type 1 als type 2 diabetes. Het suggereert dat het falen van zowel de β- als α-celadaptatie kan bijdragen aan de ontwikkeling van diabetes. Voor toekomstig onderzoek naar veranderingen in de β-celmassa is het daarom relevant om ook de α-celmassa te bestuderen.

Model	Soort	α-celmassa	Ratio α- tot β-cellen
Obesitas	Mensen	↑	=
HVD	Muizen	↓	=
GLP-1RA	Muizen	↓	=
HVD + GLP-1RA	Muizen	↓	=
KD	Muizen	↓	↓

Tabel 3. Veranderingen in de α-celmassa en de ratio α- tot β-cellen in obese vs. slanke mensen (Obesitas), HVD-gevoede vs. controle muizen (HVD), liraglutide-behandelde vs. controle muizen (GLP-1RA), liraglutide-behandelde HVD-gevoede vs. controle muizen (HVD + GLP-1RA), KD dieet-gevoede vs. controle muizen (KD). HVD = hoog-vet dieet, GLP-1RA = glucagon-like peptide 1 receptor agonist, KD = ketogeen dieet.

Onderzoek naar adaptatie mechanismen in humane eilandjes

Voor het bestuderen van β- en α-celadaptatie in humane eilandjes zijn we momenteel afhankelijk van histologische analyses van biopten die genomen worden bij een autopsie of na pancreatectomie. Dit is altijd een momentopname. Diermodellen bieden meer mechanistisch inzicht omdat β- en α-celadaptatie in reactie op metabole veranderingen op een gecontroleerde wijze en op verschillende tijdspunten bestudeerd kunnen worden. In aanvulling hierop zouden in vitro biotechnologische platforms een uitkomst kunnen bieden. Hierbij worden primaire humane eilandjes cellen gebruikt om de invloed van verschillende metabole stimuli en factoren

op de functie en overleving van eilandjes te bestuderen. Zo zouden we nieuwe mechanismen kunnen identificeren die betrokken zijn bij de adaptatie van β- en α-cellen. Momenteel is er echter geen assayplatform beschikbaar waarmee adaptatie van humane eilandjes gemeten kan worden. Daarom hebben we drie 'high-throughput' kweekplatformen opgezet met humane eilandjes om hiermee de functie van β-cellen te meten (**hoofdstuk 7**): (i) intacte humane eilandjes, en (ii) cellen afkomstig van uit elkaar gehaalde eilandjes gekweekt in een monolaag op extra-cellulaire matrix of (iii) gereaggregeerd in humane eilandjescelclusters. De uit elkaar gehaalde eilandjescellen kunnen efficiënt worden getransduceerd met adeno- of lentivirussen. Uit elkaar gehaalde eilandjescellen die in clusters werden gekweekt leken qua functie het meest op intacte humane eilandjes. De platforms kunnen ook gebruikt worden om andere aspecten van de humane eilandjesadaptatie te bestuderen, zoals α-celfunctie, β- of α-celproliferatie en β- of α-celoverleving. De drie kweekplatforms kunnen bij toekomstig onderzoek gebruikt worden voor het screenen van virale shRNA-collecties of collecties van chemische moleculen om zo nieuwe mechanismen te identificeren die betrokken zijn bij β- en α-celadaptatie van humane eilandjes.

Curriculum vitae

The author of this thesis, Johanne Hendrike (Rianne) Ellenbroek was born on the 9th of September 1985 in Zwolle, The Netherlands. She completed her pre-university education at the Greijdanus College, Zwolle in 2003. That same year she started her study in Biomedical Sciences at the University of Leiden, Leiden, The Netherlands. During her studies she conducted a research project at the department of Thrombosis and Hemostasis of the Leiden University Medical Center (LUMC) under the supervision of dr. A.Y. Nossent and prof. dr. H.C.J. Eikenboom. After obtaining her bachelor's degree, she started the master's program of Biomedical Sciences at the University of Leiden. She performed a research project at the Netherlands Institute for Neuroscience in Amsterdam under the supervision of prof. dr. A. Kalsbeek and in collaboration with prof. dr. E.J.P. de Koning, departments of Nephrology and Endocrinology, LUMC. The author received a scholarship to follow a master's course at the Karolinska Institutet in Stockholm, Sweden. A second master's research project was conducted at Unilever Research & Development, Vlaardingen under the supervision of K. Wieland. She obtained her master's degree *cum laude*. In 2009 she started her PhD program, of which the results are described in this thesis, at the department of Nephrology of the LUMC under the supervision of prof. dr. E.J.P. de Koning, prof. dr. T.J. Rabelink and dr. F. Carlotti. Part of this project was performed at the biotechnology company Galapagos in Leiden, under the supervision of dr. R.A.J. Janssen. In 2013, the author received a 4-year Junior Diabetes Fonds Fellowship to study the role of protein kinase A signal transduction in β-cell survival. In the context of this fellowship she started as a postdoctoral fellow in the groups of dr. B. Wicksteed and prof. dr. C.J. Rhodes at the Kovler Diabetes Center of the University of Chicago, Chicago, USA in 2014.

List of publications

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