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Influence of Islet-derived Factors in Islet Microcirculation and Endocrine Function

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Abstract

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Diabetes mellitus is a disorder with complex pathology and is frequently associated with vascular complications. In the islet micro milieu locally generated factors may affect both the physiology and the morphology of the tissue. This thesis examines the impact of four different islet-derived factors; thrombospondin-1 (TSP-1), ghrelin, Cocaine and amphetamine regulated transcript (CART) and irisin, and how they influence the endocrine pancreas.

TSP-1 is an angiogenesis inhibitor. Islets from TSP-1 deficient mice were hypervascular, but with normal endocrine mass. Beta-cell dysfunction was present in islets of TSP-1 deficient mice, both *in vivo* and *in vitro.* When trying to reconstitute TSP-1 in islets of TSP-1 deficient animals through a transplantation model, adult islets failed to recover, showing the importance of TSP-1 for glucose stimulated insulin secretion and thereby glucose homeostasis.

Ghrelin inhibited glucose stimulated insulin secretion and decreased the islet blood flow, while the ghrelin receptor antagonist GHRP-6 in fasted, but not fed, rats increased the islet blood flow fourfold and improved the peak insulin response to glucose. The ghrelin receptor GHS-R1α was identified in the alpha cells and the islet arterioles.

CART selectively reduced the islet blood flow in the pancreas, and this effect was unaltered by simultaneous administration of an endothelin-A receptor antagonist. CART administration did not affect insulin release, neither in insulin release from isolated islets or in an intravenous glucose tolerance test.

Irisin was confirmed located within the pancreatic islets predominately in the alpha-cells. Irisin reduced islet and white adipose tissue blood flow. Irisin was secreted as a response to increased glucose concentrations *in vivo*. Irisin had no direct effect on insulin secretion.

In conclusion, all factors investigated proved to have roles locally in the endocrine pancreas. TSP-1 deficiency caused vascular morphological alterations, and chronic β-cell dysfunction. Ghrelin, CART and irisin all decreased islet blood flow. Ghrelin acted directly through its receptor GHS-R1α in islet arterioles, thereby restricting the insulin response to hyperglycemia, whereas for CART and irisin the specific mechanism continues to be unknown, without identification of a receptor. In order to reach full physiological understanding, the receptors for CART and irisin need to be identified. All four islet-derived factors hold potential for the treatment of type 2 diabetes.

Keywords: diabetes mellitus, pancreas, blood flow, islet vascularity, islet-derived, TSP-1, ghrelin, CART, irisin

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Eat when you can, sleep when you can, and don't mess with the pancreas

- The three Basic Rules of Surgical Training

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I. Drott CJ*, Olerud J*, Emanuelsson H, Christophersson G, Carlsson P-O, Sustained Beta-Cell Dysfunction but Normalized islet Mass in Aged Thrombospondin-1 Deficient Mice, *PLoSOne 2012:7(10):E47451*

II. Drott CJ, Franzén P, Carlsson P-O, Ghrelin in rat pancreatic islets decreases islet blood flow, *Am J Physiol Endocrinol Metab 2019: (317):E139-E146*

III. Drott CJ, Norman D, Espes D, CART decreases islet blood flow, but has no effect on total pancreatic blood flow and glucose tolerance in anesthetized rats, *In Press (Peptides)*

IV. Drott CJ*, Norman D*, Carlsson P-O, Espes D, Irisin is present in α cells and decreases islet blood flow, *Manuscript*

*Equal contribution

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Contents

Abbreviations

AMPK	Adenosine monophosphate activated protein kinase
ANOVA	Analysis of variance
BAT	Brown adipose tissue
BQ-123	Selective ET_A endothelin receptor antagonist
cAMP	cyclic Adenosine monophosphate
cGMP	Guanosine 3' 5' – cyclic phosphate
CART	Cocaine and Amphetamine Regulated Transcript
CBF	Colonic blood flow
CNS	Central nervous system
DBF	Duodenal blood flow
DM	Diabetes mellitus
EC	Endothelial cells
ELISA	Enzyme linked immunosorbent assay
eNOS	Endothelial Nitric Oxide synthase
ERK	Extracellular signal regulated kinase
ET-1	Endothelin-1
ET_A	Endotelin-1 receptor type A
ET_B	Endotelin-1 receptor type B
FNDC5	Fibronectin type III domain-containing protein 5
GHRP-6	Growth hormone releasing peptide 6
GHS-R1α	Growth hormone secretagogue receptor 1α
GLP-1	Glucagon-like peptide-1
GLUT-4	Glucose transporter type-4
GSIS	Glucose stimulated insulin secretion
IBF	Islet blood flow
ipITT	Intraperitoneal insulin tolerance test
IRS-1/2	Insulin receptor substrates
ivGTT	Intravenous glucose tolerance test
KO	Knock Out
MAPK	Mitogen activated protein
MMP-2/9	Metalloproteinase 2/9
NO	Nitric Oxide
PBF	Pancreatic blood flow
PCR	Polymerase chain reaction
PET	Positron emission tomography
PGC-1α	Peroxisome proliferator-activated receptor-γ coactivator 1α

PKA	Protein kinase A
PDGF	Platelet derived growth factor
PKB/Akt	Protein kinase B
RBF	Renal blood flow
SEM	Standard error of the mean
SMC	Smooth muscle cell
T1D	Diabetes mellitus type 1
T2D	Diabetes mellitus type 2
TGFβ-1	Transforming growth factor beta-1
UCP-1	Uncoupling protein-1
WAT	White adipose tissue

Introduction

Preclinical studies of islet morphology and physiology have contributed extensively to the understanding of diabetes mellitus (DM) and the multi factorial background of the disease. The endocrine parts of the pancreas have been known for over 150 years, and all from measurements of blood flow [2, 3] to the discovery of different locally produced hormones and peptides [4-9] have contributed to the knowledge of this important organ. The pancreatic islets are much more vascularized than the exocrine pancreas, and the regulation of basal and stimulated blood flow is modified by local endothelial mediators, the nervous system as well as by gastrointestinal hormones [10]. This thesis elucidates the role of four different locally produced factors, for the function of the endocrine pancreas. In the long run, this information may contribute to find new treatments for diabetes, improving the function of the pancreatic islets.

The Pancreas and Islets of Langerhans

The pancreas is a visceral organ weighing approximately 50-100 grams in an adult. The pancreas develops from two parts of the embryonic gut epithelium, the ventral part and the dorsal part. This creates the three anatomical structures, where the ventral anlage becomes the caput (head), with close proximity to the duodenum, and the dorsal part becomes the corpus (body) and the caudal (tail) region. The different regions of pancreas have separate blood supply. The superior mesenteric artery supplies the caput region (≈ 30% of the gland), while the corpus and caudal regions are supplied by the coeliac artery (≈ 70% of the gland). In pancreatic development, blood vessels act as key inducers, specifically for the development of the dorsal pancreas, which lies close to the dorsal aorta [11, 12]. The pancreas consists of an exocrine and an endocrine component where the endocrine component, the pancreatic islets, constitutes about 1-2 % of the organ. The exocrine pancreas has a crucial role for digestion and secretes many enzymes via the pancreatic duct into the duodenum. The exocrine part is not further taken into account in this thesis.

The islets of Langerhans were first described in the rabbit pancreas in 1869, discovered by the medical student Paul Langerhans. By then, their function was still unknown [13]. In 1893, Edouard Laguesse, after noticing

the same structure yet again, suggested them to be involved in the endocrine function and in the control of the blood glucose levels, and he decided to name the "rediscovered" islets in the human pancreas after their original discoverer Langerhans [14]. A healthy human has approximately 3.2-14.8 million islets and each islet has about 5000 endocrine cells as its endocrine tissue [15, 16]. The size of an islet varies from just a small cluster of cells up to 0.5-1 mm. The islets of Langerhans are composed of five major endocrine cell types, where the fifth type, the ε-cell, the central player in Paper II**,** is the most recently discovered. Since its discovery, the ε-cell has been added to the four, long known, classical cell types producing insulin, glucagon, somatostatin and pancreatic polypeptide, respectively [17]. However, also other less recognized hormones are being produced in the endocrine pancreas by these and other cells, e.g. islet amyloid polypeptide, 5-HT, apelin, CART, and irisin, of which the latter two are investigated in Paper III and IV. Inside the pancreatic islets there are, besides the endocrine cells, several other cell types, such as endothelial cells (ECs) producing e.g. thombospondin-1 (TSP-1), (Paper I), dendritic cells, fibroblasts, macrophages and nerves.

The majority of the endocrine cells, the insulin-producing β-cells, comprises 50-75 % of all islet cells, α-cells, that produce glucagon, comprise around 15-20 %, while somatostatin-producing δ-cells constitute almost ten percent. Somatostatin inhibits both insulin and glucagon secretion, presumably mainly through local interactions. Polypeptide (PP) -cells constitute less than five percent of the pancreatic islet endocrine cells, and counteract the secretion of exocrine substances. The ε-cells constitute about one percent, and produce ghrelin [17].

Blood Vessels, Blood Flow and Islet Microcirculation

During the development of the pancreas, blood vessels are pivotal [12, 18, 19]. The pancreas development is tightly connected to endothelial cells (EC) and even during adulthood, blood borne, local and paracrine signals from the EC are essential for endocrine cell differentiation [20], maintenance of β-cell function [21], and during certain conditions even expansion of the adult β-cell mass [22]. Islet EC support islet function and contribute to enhanced glucose-stimulated insulin release and diminished internal degradation of insulin in the cells [11, 23], and with aging, recent studies have noticed a correlation for one of this factors, platelet derived growth factor (PDGF), with β-cell proliferation and function in aging, being an example of these factors' supporting function throughout life [24, 25].

The pancreatic islets in the adult are highly vascularized by a dense capillary network, and the endocrine pancreas is one of the most perfused organs in the body. The islet capillary network is approximately five times denser in islets than in the surrounding exocrine pancreatic tissue [26], and the vascu-

lar density is close to 10 % [27]. This likely reflects the importance of glucose sensing, the requirements of the organ for a rich supply of nutrients and oxygen, as well as its need for rapid and effective transport of metabolites and hormones into the blood stream [28]. It may also reflect the local importance of EC to regulate β-cell differentiation and growth [29, 30].

The capillary network has a glomerular-like angio architecture, which means that in the islet, each β-cell is surrounded by one to three islet EC, and therefore these cells by necessity are exposed to each others products [31]. This assures that no portion of an islet is more than one cell away from arterial blood [31-33]. The mean diameter of endocrine capillaries is significantly greater than exocrine capillaries, and the capillaries in the islets possess ten times more fenestrae than the pancreatic exocrine capillaries [22, 26]. In total these fenestrated capillaries constitute 7 % to 8 % of the islet volume [26]. The high amount of fenestrae is remedied by the local production of vascular endothelial growth factor-A (VEGF) that originates from the β-cells [34]. The number of blood vessels varies depending on the size of the islet. Small islets (diameter <150 μm) receive their blood supply from one arteriole and drain through numerous efferent capillaries into a basket-like collecting network around the islets, which subsequently drains into intralobular venules, thus they seem integrated into the exocrine capillary system. Large islets (diameter >150 μm) possess one-three afferent arterioles, and efferent capillaries drain into post capillary venules at the edge of the islets, which then empties into intralobular veins which eventually empty into the portal vein [35].

Pancreatic and islet blood flow have been widely studied in rodents with many different techniques [3] of which the use of non-radioactive microspheres [36] is now considered the gold standard. In the studies using the microsphere technique, the islet blood flow (IBF) has been found to be, when corrected for weight, 5-6 ml X min^{-1} X g $islets^{-1}$, which is one of the highest blood flows in any organ in the body.

The islets normally receive 7-15 % of the whole pancreatic blood flow (PBF), despite the islets contributing only ~1-2 % to the pancreatic volume [2, 37, 38]. Also, the oxygen tension (PO_2) of large superficial pancreatic islets has been reported to be ≈40 mmHg, which is much higher than in other visceral organs [39, 40]. This might reflect high needs of oxygen for adequate glucose sensing and metabolism of the pancreatic islets [41]. The blood perfusion normally matches the metabolic needs for insulin release [26, 42, 43]. In the perfusion of the endocrine pancreas the dominating idea is that β-cells are prioritized and perfused before the other endocrine cells [44, 45], in the order beta-alpha-delta; B-A-D. However, an opposite pattern with a blood flow from the periphery (mantle) towards the center (core) has been proposed [32, 45], and in a more recent study both patterns of blood flow is present [44].

Throughout the body, blood flow regulation is performed mainly in the proximal blood vessels of the microcirculation. In most vascular beads the arteriolar smooth muscle cells (SMC) are the most important sites of regulation. In human pancreas, the SMC are located in the intra islet arterioles, while in rodents, they are located mainly outside the islet [29]. Arteriolar SMC are therefore the major site for blood flow regulation in both the endocrine and the exocrine pancreas [10], and this is essential for both vasoconstriction and vasodilation. The blood perfusion of the islets is regulated separately from that of the exocrine parenchyma, this regulation occurring at a pre-capillary level, in the arteriole, anatomically separated from islets, and glucose, incretins and fatty acids cause a preferential or selective increase in IBF [29, 32, 43, 46-49]. These processes are highly affected by arteriolar SMC producing local endothelium-derived vasoactive substances that play a substantial part as the actual mediators of IBF, particularly nitric oxide (NO), endothelin-1 (ET-1) and adenosine [10, 49-51].

The regulation of the basal and stimulated blood flow in the endocrine pancreas has traditionally been considered to occur through neural and endocrine factors, for the latter mainly via gastrointestinal hormones. However, in the recent three decades more knowledge about islet-derived factors and the local pancreatic environment has evolved, and today we know that an endocrine-vasculature interaction, through endothelial mediators, is present and that receptors for different neuro- and vasoactive peptides/substances in the islets play a modulating effect on the insulin secretion [32, 43, 51, 52]. The islets are specifically sensitive to endothelial mediators, as mentioned especially the effects of NO, which is a prerequisite to maintain the high basal IBF [53]. Human islet endocrine cells have sparse contact with autonomic axons. Instead, the regulation is run by the sympathetic axons, prioritizing to innervate blood vessel SMC, that is, mainly metarterioles and arterioles. In this way sympathetic nerves is suggested to modulate islet hormone secretion in human islets by a direct effect on IBF, and not through a binding specifically to the endocrine cells [10].

Diabetes mellitus

DM is a global disease increasing fast in number of affected individuals, especially during the last three decades. There were in 2017 an estimated 463 million individuals with DM globally, a number which is estimated to increase to 700 Million in 2045 according to the International Diabetes Federation [54].

Diabetes is Greek and means “to pass through”, whereas mellitus is Latin for “sweet”. It is a heterogeneous disease, and arises due to a mixture of genetic load and environmental risk factors causing inflammation, autoimmunity and metabolic decompensation. DM exists in two major forms, type

1 diabetes (T1D) and type 2 diabetes (T2D). T1D usually affects younger individuals and is an autoimmune disease, resulting in abolished insulin production from the pancreas caused by an almost complete autoimmune destruction of the pancreatic islets [55]. In T2D, most often with a debut at higher age, the sensitivity for insulin is less due to peripheral insulin resistance, leading to a mismatch where more insulin is needed than produced. The hyperglycemia, insulin resistance, and β-cell dysfunction in T2D is often associated with obesity. For both T1D and T2D hyperglycemia becomes overt. In T2D, with time, there is also a reduction in β-cell mass (up to approximately 50%) [56], and hence decreased insulin secretion. There are also other types of diabetes present with a mixture of symptoms; i.e. maturity onset diabetes of the young (MODY) and late autoimmune diabetes in adults (LADA). Another special form is gestational diabetes, a condition where a pregnant woman, without prior diabetes, develops hyperglycemia during pregnancy, due to a temporary higher demand for insulin, and a pancreas unavailable to adopt to this new challenge. The same individuals are prone to later in life develop T2D. Of all diabetes patients T2D marks for approximately 90% and T1D for 5-10% [54]. The disease is multifactorial, and T2D is characterized by a range of metabolic disturbances; such as hyperinsulinemia, enhanced hepatic gluconeogenesis, impaired glucose uptake, metabolic inflexibility, and mitochondrial dysfunction.

In the last decades there has been a dramatic improvement in the treatment of the disease. However, DM still causes an impaired life expectancy in both T1D and T2D patients, even despite modern treatment, where DM often leads to severe complications [57].

Complications and Treatment of Diabetes Mellitus

Diabetic patients suffer both acute and chronic complications. The most common acute complications are hypoglycemia and diabetic ketoacidosis (DKA). DKA accounts for half of all deaths in young patients with T1D. DKA is caused by an absolute, or relative insulin insufficiency making it impossible to use glucose as a fuel source. This increases lipolysis and the serum levels of fatty acids. Ketone bodies are formed from this fat in order to maintain energy supply to the brain, and the ketone bodies decreases the pH level of blood leading to the classic triad of DKA; ketonemia, acidosis and hyperglycemia [58]. This situation could be lethal.

The chronic complications of DM can be divided into micro- (retinopathy, nephropathy, neuropathy and foot ulcers) and macro vascular complications (stroke, myocardial infarction and heart failure), thus DM could basically affect every organ of the human body. Retinopathy is often the first long-term complication to occur in T1D patients. For this reason, all T1D patients in Sweden are included in a screening program with retina examina-

tion every one or two years, to give one example. By improvement in glycemic control, and by intensive diabetes treatment, a preventing effect for these complications is noticed [59], but despite adequate treatment, patients with T1D still have a reduced life expectancy [60]. Concurrently, treatments plays an extremely important roll, e.g. for each 1% (10 mmol/mol) fall in HbA_{1C} concentration it leads to an estimated fall of 30% in the risk of microvascular complications [59, 61]. Over all, DM affects the components of the vascular wall. This endothelial dysfunction characteristic of DM leads to decreased bioavailability of NO [62, 63]. In addition DM causes dysfunction of vascular SMC [64], something that could further accentuate the effects of diminished NO production. The dysfunction of SMC and the endothelium is probably the major reasons to the micro- and macro vascular complications of DM, and thus, the need for further research in the field of vascularity and diabetes is highly needed.

A corner stone in the treatment of DM is to replace the diminished endogen insulin with exogenous insulin administration. Another part is to improve the insulin sensitivity and to minimize the glucose load, but for T1D and for severe T2D cases, for a complete treatment, addition of insulin, in some way, is needed. The insulin treatment was discovered after experiments by Fredrick Banting and Charles Best in 1921. Today, new insulin formulas and technical devices have improved the treatment, where continuous glucose monitoring and close loop systems play a more prominent roll, but at a basic level, treatment is still performed through daily insulin injections. Another branch of treatment is through transplantation of endocrine tissue, in the form of either whole-organ-pancreas transplantation, or islet cell transplantation. This is a possibility for a small number of patients where, despite intensive treatment, their clinical situation shows extreme glycemic variability and often repeated hypoglycemic episodes. This group of patient might be beneficial of transplantation. However the clinical utility is limited because of the need for life-long immunosuppressive treatment, and the adverse effect it means with increased risks of infections, renal failure and malignancies [65, 66].

For T1D, insulin replacement is absolute, while for T2D, at least in the early treatment, focus is on improving the insulin sensitivity and the remaining insulin producing function. Therefore, preservation of liver and islet function is a key strategy for the management of T2D, which e.g. is recently argued in an editorial comment by Leung [67] to be characteristics of irisin. Somewhat similar thoughts have been used regarding incretins. Incretins are gut-derived hormones able to increase glucose stimulated insulin secretion (GSIS) after a meal. They exert effect on a receptor that induces cyclic Adenosine monophosphate (cAMP) formation, which in turn increases the intracellular calcium concentration and enhances the exocytosis of insulin-containing granules, thereby potentiating only glucose-stimulated, and not basal insulin secretion [68]. More studies are needed, but it seems that in-

cretins also improve IBF, while not affecting PBF, and that their vascular effects may modulate hormone release and be beneficial during impaired glucose tolerance [10].

The specific association between islet vasculature and endocrine function is the main theme of this thesis. The treatment of DM needs to improve to normalize metabolic control, and so far many local islet factors have indicated promising characteristics. This thesis attempts to contribute with more knowledge to this interesting field.

Angiogenesis

There are two ways in which blood vessels are formed, through vasculogenesis or through angiogenesis [69-72]. In the embryo, and to a smaller extent in the adults, blood vessels originate from angioblasts, a process known as vasculogenesis, while in adulthood, the most common way is through angiogenesis [73]. Angiogenesis involves continued expansion of the vascular tree as a result of EC sprouting from pre-existing blood vessels, as well as ingrowth of transcapillary tissue pillars into existing blood vessels, known as intussusception. This is repeated many times in a mature animal, most often occurring in wound healing.

The angiogenic process begins with vasodilation of blood vessels and EC activation together with an angiogenic stimuli. This results in increased vascular permeability and blood flow. The increased levels of NO have a role in this as the main endothelial-derived relaxing factor. ECs start to migrate and proliferate from the dilated vessels, where pro angiogenic factors such as VEGF, angiopoetin-2 and proteinases interplay [20, 74, 75]. The migration moves towards areas with low oxygen tension since the hypoxic cells there secrete the pro-angiogenic factors. To stabilize the newly formed blood vessels the ECs recruit supporting mesenchymal cells through its production of PDGF [76]. The ECs and micro vessels are further stabilized through a mechanism involving transforming growth factor beta (TGFβ -1) and angiopoetin-1. Normally ECs replicate slowly due to a close balance between positive and negative regulators of angiogenesis in tissue. Under normal physiological conditions these regulators are in equilibrium and no angiogenesis takes place [77]. Important angiogenic factors are VEGF, angiopoetins, PDGF, matrix metalloproteinase-9 (MMP-9) and fibroblast growth factor-2 (FGF-2), and angiostatic factors include angiostatin, α1-antitrypsin (α1-AT), endostatin and TSP-1 [78, 79]. If this regulation is set out of control, the uncontrolled state could lead to severe pathological conditions, including e.g. hemangioblastoma, ischemic vascular disease, ophthalmic and rheumatic diseases, psoriasis and tumor growth [80]. However, there are also certain physiological conditions, such as pregnancy and wound healing, where angiogenesis is present and plays an important positive role [81].

Islet Angiogenesis

The islets express several growth factors that induce angiogenesis, including mainly VEGF-A, but also other members of the VEGF-family [82]. VEGF-A is constitutively expressed in pancreatic β-cells in humans, rats and mice [34, 82, 83]. In studies of animals lacking islet VEGF-A expression, the islets have continuous instead of fenestrated, capillaries [28, 82], moreover, the number of capillaries are much fewer. In adulthood islet endothelial cells do not replicate which is due to the counteraction of VEGF effects by several negative angiogenesis regulators [84]. VEGF-A is also a key molecule in regulating the balance between the density of the blood vessels and the islet cell mass, during pancreatic development [11, 34].

TSP – 1

TSP-1 is an extracellular matrix bound glycoprotein, which was the first naturally occurring inhibitor of angiogenesis described [85]. TSP-1 got its name, since it was released when platelets were stimulated with thrombin [86, 87]. TSP-1 in islets is more or less exclusively produced by the endothelium, and of importance for islet morphology and β-cell function [9, 88, 89].

TSP-1 is one of five thrombospondins, which all are matrix glycoproteins. TSP-1 is the glycoprotein with the most prominent and documented effect of the five. TSP-1 was first discovered as a protein stored in α-granules, but has later been observed in many different processes, involved in platelet aggregation, inflammatory responses and regulation of angiogenesis during wound repair and tumor growth [90]. In activated platelets, TSP-1 release participates in the blood clot formation together with fibrin, plasminogen, urokinase and histidine-rich glycoprotein. It also takes part in immune responses by mediating contact between platelets, and leucocytes by recognition of apoptotic neutrophils by macrophages [91] and indirectly by activation of TGFβ -1 [9, 92]. TSP-1 also protects β-cells against antioxidative stress, induced by lipotoxicity where TSP-1 regulates PKR-like ER kinase – nuclear factor erythroid 2 related factor 2 (PERK-NRF2) signaling [93]. However, TSP-1 is mainly known for its anti-angiogenic properties [94], since it induces apoptosis selectively in activated EC, i.e. those that are forming new blood vessels, but not in quiescent endothelium [95]. TSP-1 has even more anti-angiogenic properties and blocks the mobilization of pro-angiogenic factors, such as MMP-9 and VEGF, and inhibits their access to co-receptors on the endothelial cell surface [96].

The absence of TSP-1, in neonatal pancreatic islets, causes hypervascular and hyperplastic islets [88]. The explanation to this is the normal ability of TSP-1 to activate TGFβ -1 [9], a factor with an important role in pancreatic

islet morphogenesis, where it controls the pancreatic development by regulating the activity of metalloproteinase 2 (MMP-2) [97]. TSP-1 deficient mice show an almost normal morphologic phenotype except for the pancreatic islets [9]. Except the changes in vascularity, TSP-1 deficient mice also have an impaired pancreatic islet function, and become markedly glucose intolerant with decreased GSIS and decreased capacity for (pro) insulin biosynthesis, despite having an increased β-cell mass [88].

Ghrelin

The gastrointestinal peptide ghrelin was discovered by Kojima et al in 1999 [4]. This discovery was derived from the knowledge of an existing GHS-R receptor that was successfully cloned [98, 99], and an endogenous ligand was suggested to exist for this receptor. The name springs from *ghre*, the Proto Indo-European rot of the word *to grow*.

Ghrelin is generated by proteolytic cleavage of the 117 amino acid preproghrelin precursor encoded by the gene GHRL. The purified ligand is a peptide of 28 amino acids, in which the serine 3 residue is O-n-octanolyated through a unique post-translational event catalyzed by ghrelin O-acyltransferase (GOAT) [100]. This octanoyl modification is necessary in order to reach the active state [4, 100]. Human ghrelin is similar to rat ghrelin except for two amino acids. Of the total ghrelin levels in human plasma, approximately 10% is in the active, acetylated, form [101].

Initially, ghrelin production was found to be located to the stomach [4, 102]. More precise, the gastric source identified was the A-like cells in rats and the P/D1-cells in humans [103, 104]. Approximately 70% of the circulating ghrelin originates from the stomach [105]. The importance of the stomach for ghrelin production was proven by the observations that rats, after fundectomy, only had 20 % remaining ghrelin levels [106]. The situation in humans, however, is somewhat different where 35-45 % remains after a total gastrectomy [105]. Although mainly produced in stomach, ghrelin is synthesized in many other organs. Ghrelin has been detected in hypothalamus [4, 107], pituitary gland [108], pulmonary neuroendocrine cells [109], cartilage [110], adrenal glands, kidney, placenta, testis, ovary, brain, intestine, cardiomyocytes, blood vessels [107] and, of specific interest, in the pancreas [17].

The ghrelin cell type, the ε-cell, was first discovered in pancreas in 2002 through immunohistochemistry [17] and radioimmunoassay [111]. In a later study on islet cells during development [112], ε-cells were found to be originated from the duct epithelium, like islet cells in general, and that they proliferate perinatally. ε-cells share cell lineage with PP-cells and α-cells, however ghrelin has been confirmed to be expressed in a separate cell type, and this has been supported by the findings that no co-localization with any other

"classical" pancreatic hormone is present [17, 112-114]. The ε-cell is since then counted as the fifth islet cell type. Approximately 3-5 ε-cells are found in each islet in humans [17].

The pancreatic production of ghrelin was further confirmed by comparing the levels of acetylated ghrelin in the pancreatic vein (splenic vein) and artery (celiac artery) of anesthetized rats, where the levels of acetylated ghrelin was eight times higher and the level of des-acetylated ghrelin three times higher in the vein compared to the artery, interpreted as ghrelin being produced in the pancreas [115]. The localization of the ghrelin cells is species dependent, where humans have ghrelin cells in the periphery of the pancreatic islet co-localized with glucagon expressing cells, whereas in rats, ghrelin immunohistochemistry detected ghrelin in the central portion of the islet, as well as in the islet periphery [116].

During embryologic development ghrelin cells constitute ≈ 10 % of all islet cells. The amount decreases down to approximately 1 % in adults [17]. A similar relationship has been found for ghrelin mRNA, where peak ghrelin levels are reached at week 14 of gestation with lower levels in pancreas of adults [117]. Ghrelin cells are much more numerous in the foetal pancreas than the foetal stomach, suggesting that the pancreas is the major source for ghrelin production during foetal development [118].

The Growth Hormone Secretagogue Receptor

The Growth Hormone Secretagogue Receptor (GHS-R1α), physiologically active as the receptor for ghrelin, consists of 366 amino acids for a classical full-length G protein-coupled receptor with seven transmembrane domains [98, 119]. The GHS-R1α belongs to a family of receptors operating via the Gq phospholipase C (Gq-PLC) pathway [98]. Other signaling pathways involved are the PI3K pathways involved in the activation of ERK1/2, and through a subunit of GHS-R1α, activation of the PLC-protein kinase C pathway and Raf-MEK-MAPK, occurs [120, 121]. Ghrelin also has a direct effect on the tyrosine kinase receptor via β and γ subunits, which leads to activation of MAPK through the same, Ras-Raf-MEK pathway [122]. Furthermore, ghrelin exerts its effect in various cells through stimulation of cAMP-mediated PKA pathways [123]. Ghrelin needs to be acetylated in order to bind to GHS-R1α [124].

Studies for the localization of GHS-R1α have used mRNA techniques and polymerase chain reaction (PCR), with the highest level found in the hypothalamus and in the pituitary [107], in neurons in the arcuate nucleus [125], and in blood vessels and heart [107, 126, 127], with smaller expression sites identified in the thyroid gland, pancreas, spleen, myocardium and adrenal gland [107]. Double immunochemistry revealed a co-localization of GHS-R1α with glucagon-immunoreactivity and to some extent with insulin-

immunoreactivity in rat pancreatic islets indicating expression of GHS-R1α in both α- and β-cells in one study [114], or in β-cells in another [123]. The presence of GHS-R1α in islets is supported by our results in rat in Paper II.

Major Functions of Ghrelin

The major role of ghrelin is to stimulate growth hormone release and food intake. Thus, ghrelin is released to stimulate appetite. The concentration of ghrelin increases under conditions of negative energy, such as starvation, cachexia and anorexia nervosa. In contrast, circulating ghrelin concentrations decrease under conditions of positive energy balance, such as feeding, hyperglycemia and obesity [128]. One theory is that ghrelin indeed is a hormone signaling the need to conserve energy [5, 129]. Exogenously administrated ghrelin stimulates food intake in rodents [5, 130] and humans [131]. In contrast, mice genetically modified to lose ghrelin function show no effect on net food intake, but lower weights indicating a higher motor activity and energy expenditure [132, 133]. Ghrelin, as a "hunger hormone", signals gastrointestinal status to the CNS in order to adjust food intake and energy expenditure [134, 135]. The blood level of ghrelin is also shown to increase with hunger sensation, and the most common location for the GHSR-1α is the hypothalamic neurons that regulate food intake and satiety [125].

Ghrelin has a potential to modulate blood flow. The hypotensive action suggests mainly a local vasodilatory effect [136] exerted on the receptors located in blood vessels and heart [127]. Ghrelin raises the NO concentrations [137] within blood vessels and the increased NO bioactivity is the likely mechanism underlying the observed inhibitory effect of ghrelin on the ET-1 system. However, in other studies, a central effect has also been noticed. This central action by ghrelin in the brain stem, via effect on the nucleus of solitary tract (NTS), where the baroreceptor and chemoreceptor afferents terminate, suggests a role in the central cardiovascular regulation [138].

Ghrelin does also affect the GSIS, where ghrelin, acting through GHS-R1α on the β-cells [112, 139], inhibits insulin release primarily via G α_{i2} – mediated inhibition of the cAMP-PKA-pathway [140].

GHRP-6

D-Lys-Growth Hormone Releasing Peptide-6, (GHRP-6), is a synthetic antagonist of the GHS-R1α, being able to antagonize the effects of various peptidyl and non-peptidyl growth hormone secretagogue receptors in various experimental models *in vitro* and *in vivo* [141]. Originally a similar peptide, a Met^5-enkephalin analog, was found to stimulate growth hormone release from rat pituitary glands *in vitro*. The effect was specific for GH, and

through computer modeling techniques the more potent and today used hexapeptide GHRP-6 was designed and synthesized [142].

Since ghrelin has mainly been shown to inhibit insulin release, a role for a ghrelin receptor blockade (e.g. GHRP-6) might be a promising therapy in T2D, where GHRP-6 possibly could improve glucose homeostasis, something that has been demonstrated with oral administration of a GHS-R1α antagonist [143]. Additionally, since ghrelin is a hormone stimulating hunger, an effect of GHRP-6 would be a possible treatment of obesity. GHS-R1α antagonism increases plasma insulin and decreases glycaemia, showing a systemic role for endogenous ghrelin [140]. This effect is also present when administrating GHRP-6 to gastrectomized rats, a model with no ghrelin production from the ventricle. These animal displayed plasma insulin levels comparable to normal rats, which suggests that the ghrelin exclusively produced in the pancreas serves as a local regulator of insulin release, overall independent from the circulating ghrelin levels [115].

Cocaine and Amphetamine Regulated Transcript

Cocaine and amphetamine regulated transcript (CART) was initially identified as an mRNA transcript linked to acute psychostimulant use, reacting on either cocaine or amphetamine [6]. CART is a neurotransmitter and anorexigenic hormone [144, 145], and have a role in feeding behavior regulation, in maintenance of body weight, in reinforcement and reward, in the regulation of blood flow, in endocrine function [146], and in mediating the locomotor effects of psychostimulants [145].

CART is transcribed as two alternatively spliced mRNAs that are of different lengths and hence produce pro-peptides of different length, pro-CART 1-89 and pro-CART 1-102, which are pro-hormones of either 89 or 102 amino acid residues. Regardless, the active amino acids are identical in both the short and long form in the same species. In rat, both types are found, whereas in humans, only proCART 1–89 is present [147]. Therefore, pro-hormone convertases in human result in the peptides CART 42–89 and CART 49–89, while proCART 1–102 in rat results in the peptides CART 55–102 and CART 62–102 (the nomenclature is based on the first and last amino acids of the CART precursor). The predicted signal sequence is 27 amino acid residues. The mRNA splicing has no effect on the final peptide, as the active parts of the CART peptides are encoded by a sequence that lies downstream of the spliced region and is therefore identical in both pro-peptides [6, 148]. The proCART peptides undergo several processes that produce at least two known biologically active peptides, CART (55-102) and CART (62-102), used in most animal studies, and each containing three potential disulfide bridges. Despite the long and short splice variants resulting in the same peptides, the amino acid sequence differs slightly between human and rat, with

one exchanged amino acid [6, 149]. The human cDNA sequence is 80% identical to the corresponding rat cDNA, with 92% homology observed within the deduced protein-coding region. In this thesis, and in the vast majority of previous studies, the peptide CART 55-102 is used.

CART is transcribed from the *CARTPT* gene, localized to chromosome 5. *CARTPT* is expressed in the central and peripheral nervous system, as well as in many endocrine cells. CART has been localized in the pituitary [150], the hypothalamus [6], the adrenal medulla [150], in the antral gastrin producing G-cells in the stomach [151], and in endocrine and neural tissue of the endocrine pancreas [152]. The parasympathetic and sensory nerves innervate the islet, and suggest that CART interacts in the parasympathetic control of islet function, in the regulation of insulin secretion and in the stimulation of pancreatic exocrine secretion [7, 152-155]. CART is highly expressed in several islet cell types during development [152], and CART KO mice exhibit impaired β-cell function both *in vivo* and *in vitro* in islets from KO animals [156]. Moreover, humans with missense mutation in the *cart* gene are prone to develop T2D [157], indicating a role for CART in normal islet function and in pathophysiology of T2D.

During development islet CART is upregulated; in rats in almost all islet cell types, and in mice mainly in the α-cells [7, 158]. This expression peaks around birth. In humans, instead, intrapancreatic neurons and all novel islet cell types but the ghrelin cells, express CART during fetal and neonatal development [7, 152, 158]. In adult rats CART is expressed in δ cells and in a minor subpopulation of β-cells [159], in adult mice CART is mainly expressed in nerve fibers, and in a subpopulation of β-cells [7], whereas in humans CART is expressed in both α- and β-cells [158]. The levels of CART seem affected by T2D, where several experimental models of T2D, and pancreata from human type 2 diabetic patients, have shown upregulated levels of CART [7, 158, 159].

CART is considered a neuropeptide and therefore a receptor for CART peptides should most likely exist. Despite known structure and many known functions of CART peptides, the neuronal targets for CART peptides are yet not fully understood [160-162], and so far no receptor has been found.

Major Functions of CART

CART affects both the endocrine functions, as mentioned above, and the vascularity. CART has been localized to the nucleus of the solitary tract and the area postrema [163], both major cardiovascular centers. When administrating CART centrally, CART evoked an increase in arterial blood pressure in conscious rabbits [164] and in rats [165], and blocked phenylephrine-induced bradycardia in the rat [166]. CART is also released in hypothalamic-pituitary portal circulation in response to NO-induced hypotension [167].

Another finding is that CART peptide has a direct effect on the regulation of the vascular tone in cerebral arteries, through a mechanism involving ET-1 [168, 169]. Here, administration of ET-1 antagonists (nonspecific PD-145065 and ET_A specific BQ-123) blocked the normal vasoconstrictive effect of CART in cerebral arteries in rats, without having any effect when administrated alone, while the ET_b-specific antagonist (BQ-788) had no effect on the vascular response to CART [169]. However, BQ-788 had a significant effect on constriction of cerebral arteries when administrated alone. Also administration of phoshoramidon (PHO), an endothelin-converting enzyme inhibitor, attenuated the constrictor response from CART, whereas PHO alone created a mild vasodilating response.

In summary, the specific mechanism performed by CART on the vasculature is still not concluded, but an effect of CART on the vessels and their contractile machinery is the dominating theory. The mechanism involving CART and ET-1 was further evaluated in paper III of this thesis.

Endothelin-1:

Endothelin-1/A (ET-1) is the most potent vasoconstricting agent currently identified [170]. It is derived from the endothelium, where it is synthesized from the precursor proendothelin by endothelin converting enzyme (ECE). ET-1 acts primarily through the smooth muscle bound ET_A receptor to cause potent and long-lasting vasoconstriction [171]. Also an ET_B receptor is present on SMC and on EC [170]. The activation of ET_A or ET_B in VSMC results in vasoconstriction, while activation of ET_B in EC induces NO, produced through endothelial nitric oxide synthase (eNOS). Thus the functional response to ET-1 varies depending on the distribution and expression of ET_A or ET_B receptors [172].

ET-1 has previously been shown to markedly decrease total PBF and especially IBF, despite having only minor effects on systemic blood pressure, identifying that pancreatic islet vasculature is highly sensitive to exogenous ET-1, through effect on the ET_A receptor. This suggests that ET-1 act as an important regulator of PBF and IBF [173]. ET-1 is also produced in the pancreatic vasculature and predominantly in the islet cells [174, 175]. ECE responds to a high glucose level in EC and increases the level of ET-1, causing high levels of ET-1 in DM [170]. Concordingly, ET-1 is considered to potentiate glucose-stimulated-insulin-release in mice [176] and in rats [177].

Irisin

The myokine irisin is secreted following proteolytic cleavage of its precursor fibronectin type III domain-containing protein 5 (FNDC5) [8, 178, 179].

Irisin was discovered by Boström and colleagues in 2012 [8]. FNDC5 main source of production is the skeletal muscle, with the primarily synthesis from the heart muscle [178], but FNDC5/irisin is also expressed in other tissue such as adipose tissue and liver [180, 181]. Irisin has also been detected in the cerebrospinal fluid, breast and ovarian cancer cells, liver, pancreas, stomach, serum, saliva and urine in humans, and in kidney, heart, brain, liver, muscle, skin, retina, pineal and thyroid glands in rats [182].

Once released, irisin exerts its major action by increasing the expression of mitochondrial uncoupling protein (UCP1), which facilitates the conversion of white adipose tissue (WAT) into beige adipose tissue, a conversion where WAT aquires brown-adipose-tissue (BAT) -like properties and is involved in thermogenesis [182-184]. This transformation takes place through the pathway of mitogen-activated protein (MAPK) and extracellular signal regulated kinase (ERK), with the net effect weight reduction and improved glucose metabolism [185]. FNDC5 is a glycosylated type 1 membrane protein that contains an (1-28 aa) N-terminal stage peptide, a (33-124 aa) Fibronectin III domain, a (150-170 aa) transmembrane domain and a (171-209 aa) cytoplasmic tail. Irisin is activated, as a 112 amino acid protein, by exercise and peroxisome proliferator-activated receptor-γ coactivator 1-α (PGC-1α) [186]. It is capable of regulating multiple genes in response to nutritional and physiological signal in tissue, where overexpressed, like in skeletal muscle, BAT, liver and heart [181, 186].

Irisin has a 100% identical amino acid sequence among most mammalian species, suggesting a highly conserved function [8]. Irisin levels have been found to be lower in patients with T2D [187, 188], while they are higher in patients with T1D [189, 190].

Brown Adipose Tissue

BAT is mainly present in childhood but has recently created more interest due to its presence also in adults, where it was rediscovered through positron emission tomography (PET) [191-193]. Brown adipose cells could convert energy into heat and thus lead to weight loss. For this process a specialized mitochondrial protein is used; uncoupling protein-1 (UCP1). UCP1 has an ability to transport protons across the inner mitochondrial membrane, avoiding ATP synthesis and dissipating energy as heat [183]. Regulation of UCP1 is mainly at a transcriptional level, where PGC-1α plays a key roll [194]. Studies on mice lacking PGC-1α corroborate its importance for thermogenesis [195], and in fact, the expression of PGC-1α is increased by exercise in mice, rats and in human beings [196]. Further, mice with PGC-1α transgenically increased in muscle, showed improved metabolic response regarding obesity and insulin sensitivity [197]. The analyze of this animals adipose tissue further shows a significantly increased thermogenic gene program,

where their adipocytes display several classic brown adipocyte characteristics, as elevated UCP1 mRNA and protein. Irisin was suggested to be the molecule that links exercise with increased thermogenesis, and is, partly due to this, named after the Greek goddess Iris, who served as a courier among the Gods [8].

Major Functions of Irisin

Irisin is mainly counted as a myokine, which is a hormone released from the muscle into the circulation after physical exercise. Myokines could influence metabolism and modify cytokine production in different tissues and organs, and on the basis of these properties the skeletal muscles should be considered as an endocrine organ [198, 199]. Whether irisin concentrations are increased by exercise is a matter of dispute. Some studies [8, 178, 200-204] indicate a significant increase, while others doubt any positive or negative association between the two [179, 205]. Animals exposed for swimming exercise for 8 weeks had increment in serum irisin levels and reduced body fat mass, triglycerides and total cholesterol levels [206, 207], and humans exposed for long-term running exercise had significant changes in UCP1, PGC-1α and FNDC5 expression in skeletal muscle [208]. The upregulation of UCP-1 is supposed to act through phosphorylation of the p38 MAPK, resulting in weight reduction and improved glucose metabolism [185]. Irisin has also shown an ability to lower plasma glucose levels and to alter food intake in streptozotocin-treated mice [209]. Furthermore, irisin promotes glucose uptake in skeletal muscle, through improved hepatic glucose and lipid metabolism [210], and p38 MAPK-GLUT-4 translocation [185]. On the other hand, in studies on the correlation between long-time exercise and levels of irisin, UCP1 and other browning genes fail to show any correlation [186, 211, 212].

Additionally, irisin has endothelial and cardiovascular effects. Irisin increases myocardial cell metabolism, promotes cell differentiation and inhibits cell proliferation through modulating Ca^{2+} signaling and PI3K-AKT [213]. Moreover, irisin was reported to have a relaxing effect on mouse mesenteric arteries, an effect mediated by NO and a guanosine 3' 5' – cyclic phosphate (cGMP) – dependent pathway [214]. Another possible route for effect is through tyrosine kinase receptor, which phosphorylates the insulin receptor substrates (IRS-1 and IRS-2), leading to successive PI3K and protein kinase B (PKB)/Akt activation [185, 215]. Irisin administration to obese animals improved endothelial function through enhancing of NO phosphorylation in the AMPK-eNOS pathway [216]. Furthermore, peripheral and central administration of irisin was found to regulate cardiovascular activity and blood pressure [217].

In summary, increased irisin expression leads to weight loss and improved glucose tolerance [188], and irisin shows a direct stimulatory effect on GSIS both *in vivo* and *in vitro* [218]. Irisin is a thermogenic agent that serves anti-obesity and anti-diabetic functions and acts through a cell surface receptor, so far not identified. The effect is exerted mainly through sensitization of the insulin receptor in skeletal muscle and heart by improving hepatic glucose and lipid metabolism, promoting pancreatic β-cell functions and transforming WAT to BAT [219].

Aims

General Aim

The overall aim of the work presented in this thesis was to study pancreatic endocrine function and circulation, and specifically how four different local islet-derived factors might affect these parameters. The focus has been on evaluation of islet morphology, blood flow, glucose tolerance and insulin secretion through *in vivo* and *in vitro* studies. More specifically, the aims for each study were:

Paper I

To investigate the long-term morphological and physiological changes in TSP-1 deficient mice by analysis of these mice at one year of age. This was a follow-up of a previous study where TSP-1-deficient mice developed pancreatic islet hyperplasia and glucose intolerance including decreased glucose-stimulated insulin release at young age.

Paper II

To investigate the effect of ghrelin on rat pancreatic islet endocrine function and blood flow. To achieve this, the specific location of the ghrelin receptors in the endocrine pancreas and its vasculature, and the effects exerted by ghrelin and the GHS-R1α antagonist GHRP-6 on insulin release, were analyzed.

Paper III

To evaluate the effect of the peptide CART on rat pancreatic- and islet blood flow, and on the insulin secretion.

Paper IV

To focus on the myokine irisin and its effect on rat pancreatic and islet blood flow, and on the islet function. Additionally, the specific location of irisin inside the pancreas was evaluated through immunohistochemistry.

Materials & Methods:

Ethic Statements

The studies were carried out in strict accordance with the recommendations in the Guide for Care and Use of Laboratory Animals of the National Institutes of Health, Eighth edition (2011):
(**http://grants.nih.gov/grants/olaw/guide-for-the-care-and-use-of-laboratory-animals.pdf**).

All protocols and all experiments were approved by the Animal Ethics Committee for Uppsala University, Uppsala, Sweden, and specific national laws were followed, when applicable. Experiments were performed under deep anesthesia and all efforts were made to minimize suffering.

Animals (*Paper I-IV*)

TSP-1 deficient (-/-) mice were generated by homologous recombination in 129/Sv-derived ES cells implanted in C57BL/6 blastocyst. The offsprings were backcrossed (N9) to a C57BL/6 genetic background, and were generously donated by Jack Lawler (Beth Israel Deconess Medical Center and Harvard Medical School, Boston, MA, USA). Wild-type TSP-1 (+/+) (M&B, Ry, Denmark) or TSP-1 (-/-) deficient mice at 10-12 weeks or one year of age were allocated to the different studies. All mice had free access to tap water and pelleted food and were housed in a room with a 12 h light/12 h dark cycle throughout the course of the study.

For Paper II-IV the animals used were male Sprague-Dawley rats (M&B, Ry, Denmark) 12-16 weeks of age and weighing approximately 250-400 grams. They were housed in a room with a 12 h light/12 h dark cycle and with free access to pelleted food and tap water. In Paper II, when indicated, one group of rats was fasted over night, before experiments were performed. Given the technical difficulties associated with measuring IBF and the invasive nature of the method, rat is the most widely used animal model for studies with microspheres and therefore the reason for why we choose rat as an experimental model in these studies [3]. In Paper IV, additionally, pancreata from C57BL/6 mice (M&B), 10-12 weeks of age and weighing 25-30 g, were used for immunohistochemistry analyses.

Immunohistochemistry (*Paper I, II, IV*)

In Paper I, pancreata from 10-12 week old, 16 week old, and one year old wild-type and TSP-1 deficient mice were dissected free from fat and lymph nodes, weighed, and fixed in 10% (vol/vol) formaldehyde and embedded in paraffin. In order to determine the percentage of β-cells and α-cells in the different samples, sections (5 μm thick) of pancreata were prepared and stained with an antibody for insulin or for glucagon [220], and were thereafter counterstained with hematoxylin. This procedure was performed on ten or more randomly chosen tissue pieces from each animal.

To measure the endocrine fractions a direct-point-counting method [221] was used. In this method, each piece of pancreas was first investigated in microscope for the amount of intersections overlapping the tissue in order to compensate for differences in size. Intersections located over islets were thereafter counted, thereby giving us the correct fraction of endocrine tissue. The amount of islets per square mm in the sections was also counted. This was performed through division by islets into three groups depending on their mean diameter in investigated sections, big (<200 μm), medium (25-200 μm) or small (<25 μm). For each group of islets a mean value was calculated for each animal. Similarly, the fraction of α- and β-cells of the whole islet area, respectively, was measured, and from these values the fraction of islet tissue occupied by the different cell types was estimated. All analyzes were performed by using a computerized system for morphometry (ImageJ 1.2v; Nation Institutes of Health, ML, USA).

In Paper II, pancreata were fixed in 3.7 % formaldehyde in PBS or using Zinc Fixative (BD Biosciences, Pharmingen, San Diego, CA, USA) for 24 h. Fixed tissues were dehydrated, embedded in paraffin and sectioned (5 μm thick), and mounted on Polysine slides (Thermo Scientific, Braunschweig, Germany). For immunofluorescence detection, tissue sections were deparaffinised and incubated overnight at 4° C, and double stainings were performed using anti-Ghsr (1:100, rabbit polyclonal, Almone labs, Jerusalem, Israel) together with anti-CD34 (1:100, goat polyclonal, R&D Systems, Sweden), anti-insulin (1:1000, Guinea pig polycolonal, Reactionlab, Stockholm, Sweden), anti-somatostatin (1:200, mouse monoclonal, Thermo Fisher Scientific, San Diego, USA), anti-glucagon (1:500, mouse monoclonal, Abcam, Cambridge, UK), or anti-smooth muscle cell actin (1:50, mouse monoclonal, BioLegend, San Diego, CA, USA). This was followed by 1 h incubation with secondary fluorescently labelled antibodies anti-rabbit-Alexa Fluor 594 (1:300, Jackson ImmunoResearch, West Grove, USA), anti-goat-Alexa 594 (1:300 Jackson ImmunoResearch), anti-Guinea pig-Alexa 594 (1:300 Jackson ImmunoResearch) and anti-mouse-Alexa Fluor 594 (1:300 Jackson ImmunoResearch) at room temperature.

In Paper IV, pancreata from C57BL/6 mice and human donors were dehydrated, embedded in paraffin, sectioned (5 μm thick), mounted on Poly-

sine slides (Thermo Scientific), stained and examined. Irisin-, insulin- and glucagon staining was carried out by deparaffinizing the sections followed by heating in a cooker. For blocking, 3% donkey serum/PBS was used for 30 min, followed by overnight incubation with irisin primary antibody (Bioss Antibodies, MA, USA; FNDC5 polyclonal antibody, bs-8486R) in a dilution 1:80 together with insulin (Fitzgerald, Acton, MA, USA. Cat No. 20-IP30. 1:1000) or glucagon (abcam, Cambridge, UK. Cat No. ab10988. 1:500) primary antibodies at 4^0C. For secondary antibody on FNDC5 primary antibody, Alexa 594 conjugated donkey anti-mouse (Jackson ImmunoResearch Laboratories, West Grove, PA, USA. Code No. 715-585-151); on insulin, Alexa 488 conjugated donkey anti-guinea pig (Jackson ImmunoResearch Laboratories, West Grove, PA, USA. Code No. 706-545-148); on glucagon, Alexa 647 conjugated donkey anti-rabbit (Jackson ImmunoResearch Laboratories, West Grove, PA, USA. Code No. 711-605-152) were used at dilution of 1:300 and incubated for one hour. Incubation for another 5 minutes was carried out in DAPI solution.

Fluorescent immunohistochemistry images were acquired using Zeiss LSM 780 (Zeiss, Jena, Germany) confocal. Four channels were used to simultaneously evaluate nuclei, glucagon-, insulin- and irisin staining. Evaluation was done through comparing the staining of irisin and glucagon, and confirming the staining to be intra-islet, i.e. adjacent to the insulin staining.

Identification of ghrelin receptor localization in islets with PCR Analysis (*Paper II*)

In order to perform a gene expression analysis using qPCR, islet arterioles and islets were prepared separately [222, 223].

Arterioles and islets

Immediately after euthanasia native pancreata were surgically excised from male Sprague-Dawley rats under sterile conditions and transferred into cold Hank´s balanced salt solution (HBSS without $NAHCO_3$, #991750, SVA, Uppsala, Sweden) supplemented with 1 mL Penicillin/ Streptomycin 50 000 U/mL (Roche) and 125 µL 2 M NaOH. Each pancreas was surgically minced into pieces and the pieces were then digested with Collagenase type V (Sigma C9263) 1,5 mg/mL during 17 minutes vigorous shaking in a 37°C water bath. The collagenase reaction was inactivated by diluting the suspension with cold saline wash buffer (1L Ringer Acetate Baxter, 4,5 mM $NaHCO_3$ Gibco, 2,5 mM glucose, 1 mL Penicillin/Streptomycin) and then washing twice with cold HBSS. A small volume of the digested tissue sus-

pension was transferred to a petri dish with HBSS and the islets were picked with a pipette using a dissecting microscope and then transferred to a new petri dish containing culturing media (1 L RPMI-1640 Sigma R0083, 2mM L-glutamine Sigma G7513, 10% vol/vol Fetal Bovine Serum Sigma F7524, 1 mL Penicillin/Streptomycin 50 000U/ml Roche 11074440001).

Samples containing 45-50 or 300 arterioles were collected in 350 µL of lysis buffer (RNeasy Plus Micro Kit, Qiagen, Hilden, Germany), and the samples were vortexed thoroughly before storage at -80°C. Preparation of biological samples was performed twice, at different time points.

RNA Isolation

Total RNA was isolated from the rat arterioles and rat islets samples according to the manufacturer's instructions (RNeasy Plus Micro Kit, Qiagen AB). The amount and purity (OD 260/280) of the total RNA was determined using a NanoDrop 2000C spectrophotometer (Thermo Scientific).

cDNA Synthesis

The RNA was transcribed to cDNA by Superscript First-Strand Synthesis Super Mix for qRT-PCR (Invitrogen, Life Technologies, Stockholm, Sweden) according to the manufacturer's instructions. Briefly, a mix of random hexamer primers and oligo (dT) primers were incubated with the Superscript III Reverse Transcriptase enzyme mix and RNA at 25°C for 10 min, followed by 50°C for 30 min and thereafter 85°C for 5 min to inactivate the enzyme. The mixture was incubated with two units of RNaseH at 37°C for 20 min. Two independent reverse transcriptase reactions were carried out for each RNA sample. All primers were purchased from Sigma-Aldrich (see Paper II).

Quantitative Real-time PCR

The qPCR assay was performed using a Light Cycler 480 (Roche Diagnostic, Mannheim, Germany) and Light Cycler FastStart DNA Master PLUS SYBR Green I kit (Roche Diagnostic) for detection. The qPCR reactions were carried out using an initial step of 10 min at 95°C, followed by 45 cycles consisting of 10 s at 95°C, 5 s at 55°C, and elongation at 72°C, 10 s. The fluorescence was measured at the end of each cycle and a melting curve analysis was performed directly following PCR. All qPCR samples were run in duplicates. Moreover, cDNA was prepared twice from each RNA to ensure inter run specificity. In summary, each biological sample was analyzed 2 times per gene and RNA preparation. The genes of interest were growth hormone secretagogue receptor 1a (*GHRS1a*) and ghrelin (*GHRL*). The expression stability of reference gene transcripts; actin-β *(ACTb)*, hypoxanthine guanine phosphoribosyl transferase *(HPRT)* and succinate dehydrogenase complex flavoprotein subunit A (*SDHA)* was evaluated using the Normfinder software [224]. Relative mRNA expression was calculated as $2^{-\Delta Ct}$, where

ΔCt is the Ct-value for the gene studied minus the Ct-value for reference genes.

Agarose Gel-Electrophoresis

To confirm amplicon size, qPCR products were analyzed by electrophoresis using a 3% agarose gel (PCR-grade, Bio-Rad, Hercules, USA) and the bands were visualized using GelRed (Biotium, Hayward, USA) and detected using the Chemi Doc MP Imaging System (Bio-Rad).

Islet Vascular Density (*Paper I*)

Pancreatic paraffin-embedded sections were stained with the lectin Bandeiraea simplicifolia (BS-1) as previously described [220, 225], and counterstained with hematoxylin. Lectin was necessary to use, since when performed, it was the only staining for EC working on paraffin embedded tissue. In each animal, ten or more sections were evaluated. The number of stained blood vessels in the islets was quantified under a microscope by point-counting. The area of the investigated islets was determined by using ImageJ. Vascular density, i.e. the number of BS-1-stained blood vessels per measured islet area (mm^2) was calculated.

Islet Transplantation and Graft Perfusion (*Paper I*)

TSP-1 and thereby TGFβ -1 can be reconstituted in TSP-1 deficient neonatal islets by transplantation of islets into wild type TSP-1 (+/+) recipients [88]. Islets of adult TSP-1 deficient mice show a functional defect and as an attempt to reverse this, islets derived from 10-12 week-old TSP-1 deficient mice were implanted into either wild type or TSP-1 deficient mice. Likewise, wild-type islets were transplanted into either 10-12 week old wild type or TSP-1 deficient mice. For this purpose, pancreatic islets were isolated by collagenase digestion [226], and maintained free-floating in groups of 150 islets at 37° C for 5-7 days in 5 ml culture medium composed of RPMI 1640 medium supplemented with 2 mmol/L glutamine, 11 mmol/L glucose, 10% (vol/vol) fetal calf serum, 100 U/mL sodium benzylpencillate (Roche Diagnostics, Scandinavia, Bromma, Sweden) and 0.1 mg/mL streptomycin. The transplantation was performed in avertin anestheized wild-type or TSP-1 deficient C57B1/6 mice, with implantation of 150 wild-type or TSP-1 deficient islets beneath the capsule of the left kidney. GSIS of the islet grafts were evaluated one month post-transplantation [227].

The graft-bearing left kidney was removed together with part of the aorta and inferior vena cava. The ureter and the renal vein were cut, while the aorta was canulated and infused with a continuously gassed (O_2 :CO_2 (95:5)

Krebs-Ringer bicarbonate buffer (KRBH) supplemented with 2.0% (wt/vol) each of BSA (fraction V; Miles Laboratories, Slough, U.K) and dextran T70 (Pharmacia, Uppsala, Sweden). For different time periods during the infusion, the medium contained either 2.8 or 16.7 mmol/L D-glucose. The medium was administered at a rate of 1 mL/min without recycling for 60 min with a perfusion pressure of ≈ 40 mmHg. The perfusion experiment started with a 15-min period using medium containing 2.8 mmol/L glucose, which was followed by 30 min using 16.7 mmol/L D-glucose. 1.0 mL samples were collected at 14, 15, 16, 17, 18, 19, 20, 22, 25, 30, 35, 40, 45, 50, 55 and 60 min. Insulin concentrations were measured by ELISA (Mercodia, Uppsala, Sweden) and the rate of insulin secretion was calculated by multiplying the insulin concentration in the sample by the flow.

Islet perifusion (Paper IV)

Groups of 50 size-matched human islets were inserted into filter-covered perifusion chambers (Suprafusion 1000, 6 channel system, Brandel, Gaithersburg, MD, USA). The islets were perifused (200 µL/min) with KRBH supplemented with 2 mg/mL bovine serum albumin and stepwise increasing glucose concentrations. The islets were first perifused with 2 mmol/L glucose for 30 minutes to acquire a baseline secretion. Perifusion was then performed with 2.0 mmol/L glucose for 15 min followed by 20.0 mmol/L glucose for 30 min, and then again 2.0 mmol/L glucose for 15 min. Separately, potentiation of insulin release at low (2.0 mmol/L) and high glucose concentrations (20.0 mmol/L) by forskolin (1.0 µmol/L) was performed. Released irisin was analyzed by a commercially available ELISA kit (#EK-067-29, Phoenix Peptides Europe GmbH, Germany).

Blood flow measurements (*Paper I - IV*)

The blood flow measurements were performed with a technique based on microspheres [36, 37, 228, 229]. This applies the theory for indicator-dilution methods [230]. The microspheres used consist of non-deformable polystyrene, spherical particles with a standardized diameter, and the size of the microspheres being used was decided dependent on the species used and the organ that was examined [43].

The history of the microsphere technique is well described in a review article by Prinzen *et al* [1] and recently by our research group [3]. In the review article by Prinzen *et al*, the requirements for measurements of regional blood flow by microspheres are considered, and six principles need to be fulfilled (Table 1).

Table 1. *Validation criteria for measurements of blood flow with microspheres (modified from reference Prinzen et al, [1]).*

Criteria	Comment
1. Mixing in central circulation	Site of injection: atrium or ventricle
2. Complete extraction in first pass	For microspheres: diameter .12 microns
3. No separation from the blood (no 'streaming' or 'skinning')	Diameter as small as possible ('molecular microspheres')
4. Artifacts with deposited markers	Particle density not important Obstruction of vessels No measurements in capillary beds in series
5. Retention of marker in tissue	Retention of particle in tissue No leakage of tracer from particle Poisson distribution!
6. Measuring accuracy	Detection of label

By freezing followed by thawing of the pancreatic tissue, the islets become visible [36]. The microspheres within the pancreatic tissue and the pancreatic islets are then easy to count in a stereomicroscope. The microsphere technique is a reliable and reproducible method, and is considered Gold Standard for invasive measurements of blood flow.

Regarding the experimental procedure; animals were anesthetized using an intraperitoneal (i.p) injection of (Paper I) avertin (Sigma, St. Louis, MO, USA) in mice or (Paper II-IV) Thiobutabarbital sodium (120 mg/kg; SigmaAldrich Chemie GmbH, Steinheim, Germany) in rats. Deep anesthesia was confirmed by the absence of pain reaction and reflexes, and in case of a not fully anesthetized animal, anesthetic substance was added and the experiment was put on hold for a few minutes in order for the added substance to act. Animals were kept on an operating table to maintain body temperature of 36.5–38.0 ˚C.

The surgical procedures were performed under aseptic conditions and deep anesthesia as follows: For rats a tracheotomy was performed in order to maintain adequate breathing. In all animals an incision was made just above the right clavicle. Connective and adipose tissue were then pushed aside in order to expose the right carotid artery. A polyethylene catheter filled with saline solution was pushed into the vessel until the catheter tip reached the ascending aorta close to the left ventricle. The catheter was then connected to a blood pressure transducer in order to monitor blood pressure during the experiment. Next, the left femoral artery and femoral vein were exposed following a surgical incision in the thigh. After mobilizing the artery and the vein, respectively, a catheter with heparinized saline solution was introduced into each vessel. The arterial catheter was later used for collecting reference blood, and the vein was used for infusion of substances. Blood samples for analysis of blood glucose concentration by test reagent strips (Freestyle Lite;

Abbott, Deerfield, IL, USA) and of serum insulin concentration (Rat insulin ELISA; Mercodia; Uppsala, Sweden) were collected from the tail vein and from the femoral artery, respectively.

Different substances, for each corresponding paper, were infused into the catheterized femoral vein. Approximately 9 X 10^4 (for mice) and 1-2 x 10^6 (for rat) non-radioactive black microspheres (E-Z Trac; IMT, Irvine, CA, USA) were injected via the right carotid artery into the ascending aorta. To confirm the exact flow arterial blood was collected for 60 seconds, starting immediately before the injection of microspheres, and the weight of this sample was recorded. The animals were killed by cervical dislocation under deep anesthesia. Blood reference samples were heparinized and for a short time stored at +4°C before analysis in a stereomicroscope. In order to collect the appropriate organs, a laparotomy was performed, and the pancreas was removed followed by other specified organs, specific for each corresponding Paper. The pancreas and other collected tissues were dissected free from adipose tissue and weighed.

All tissue samples underwent freezing followed by thawing, and counting of intra-islet microspheres in a microscope equipped with both dark and bright field illumination was performed [37]. The reference blood collected during the experiment was transferred to glass microfiber filters with pore size of <10 µm before the microspheres therein were counted. The organ blood flow was calculated according to the formula $Q_{org} = N_{org} \text{ X } Q_{ref} / N_{ref}$ where Q_{org} denotes organ blood flow (mL/min), Q_{ref} the flow of the reference sample (mL/min), N_{org} the number of microspheres present in the organ, and N_{ref} the number of microspheres present in each reference sample. To verify an equal distribution of microspheres, an adrenal gland blood perfusion difference of less than 20 % between left and right was used as measurement for equal distribution of the microspheres, otherwise that experiment was removed from the compilation.

Glucose tolerance and insulin tolerance tests (*Paper I - IV*)

In paper I non-fasted wild-type C57BL/6 and TSP-1 deficient mice were injected with D-glucose (2,5 g/kg body weight; 300 mg/mL) into the tail vein. Glucose was analyzed at 0, 10, 30, 60 and 120 min. Insulin was collected 10 min after the glucose injection and analyzed with commercially available ELISA kits (Mercodia).

In paper II, rats were either fasted over night or normal fed. They underwent anesthesia with i.p. Pentobarbital Sodium vet (60 mg/kg; Mebumal vet. APL, Sweden). The femoral artery and vein were dissected free, and polyethylene catheters were introduced into the vessels for later blood sampling

in the artery, and for injections in the vein. Fasting was performed in some of the animals in order to increase endogenous ghrelin levels. Rats were assigned to either injection with saline or with GHRP-6. Fifteen minutes were allowed in order for the substance to act, followed by an intravenous (i.v.) injection of glucose (300 mg/mL, 2,0 g/kg) in the intravenous glucose tolerance test (ivGTT) or insulin (Actrapid 100 IU/mL, 75 IU/kg) in the i.p. insulin tolerance test (ipITT). Blood glucose measurements were performed from blood obtained from the tail vein at 0, 15, 30, 60, and 120 min. Blood for measurements of insulin concentrations was retrieved at -15, 0, 5 and 30 min from the femoral artery. The concentration of insulin was analyzed using ELISA (Mercodia).

In paper III-IV, rats were anesthetized with Pentobarbital Sodium vet (60 mg/kg, i.p.), and polyethylene catheters placed in their left femoral artery and left femoral vein. Substance dissolved in saline or saline alone (1.0 mL/h in Paper III and 2.0 mL/h in Paper IV) was infused i.v. for 60 minutes. Glucose (300 mg/mL, 2.0 g/kg) was injected (i.e. the start of the ivGTT = min 0) 30 or 60 minutes after the start of the infusion of substance. Blood glucose measurements were performed from blood obtained from the tail vein at -60, -30, 0, 5, 15, 30, 60, 90 and 120 min. During the ivGTT, arterial blood samples for analysis of samples corresponding to each paper were collected at -60, -30, 0, 5, 30 and 60 min, and later analyzed by appropriate ELISA.

All arterial samples were analyzed following a slight modification of a standardized method developed by Hosoda et al [101], where blood samples where collected in K_2 EDTA (Di-Potassium Ethylenediaminetetraacetic acid) – aprotinin tubes, rapidly cold down, and centrifuged for 5 minutes, (5000 ROW and temp –4 °C). In paper II, exclusively, the serum was then distributed to Beckman tubes, 1 mol/L hydrogen chloride (HCl) was added, 50 µL at a time, the tubes were vortexed for 1 min, and pH measured via lackmus paper test until a pH of 4 was reached. The processed serum was quickly stored at -80°C. The acidification and low temperature procedures are simple, reliable means to protect against degradation of the acylated ghrelin modification [231]. Hosoda´s method is a further development of the RAPID method [232], a standardized method that was introduced due to previous difficulties in measuring peptides. All blood samples were measured using commercially available ELISA kit. In Paper III-IV the substances were quickly stored at -80°C, without the acidification step with HCl.

Islet isolation and insulin release (*Paper II-IV*)

Pancreatic islets were isolated as previously described [222] from Sprague Dawley rats, in paper II from fed or fasted Sprague Dawley rats. After being incubated for 48-72 h, islets were transferred to one 6-well plate per animal, with each well containing a specified number of islets, and RPMI 1640 cell

medium (Sigma-Aldrich, R0883). Prior to the experiment, the RPMI 1640 cell media was supplemented with 10% vol/vol fetal bovine serum (Sigma-Aldrich, F7524), 1% vol/vol L-glutamine (Sigma-Aldrich, G7513) and 0.2% vol/vol penicillin/Streptomycin (50 000 U/mL and 50 mg/mL respectively, 11074440001, Sigma-Aldrich). For the insulin release part, corresponding substance was added to three of the wells per animal, *i.e.* the substance-group, with the other three wells constituting the control group. All islets were incubated for one hour. Next, triplicates of ten freshly isolated islets were incubated in KRBH supplemented with 10 mmol/L HEPES and 2 mg/mL bovine serum albumin and analyzed for basal (1.67 mmol/L glucose; 1 h incubation) and glucose-stimulated (16.7 mmol/L; 1 h incubation) insulin release in the presence or absence of substance (substances were added at different time of the experiment depending on each experiments unique set up). The supernatants were analyzed for insulin concentration (rat insulin ELISA, Mercodia).

Statistic analysis

Calculations were (Paper I) performed in SigmaPlot (SPSS Science Software, Erfart, Germany), and in (Paper II-IV) in Prism (GraphPad Software Inc, San Diego, CA, USA).

Parametric data with only two groups were analyzed with Student's two-tailed *t*-test for unpaired and paired observations, whereas non-parametric values were compared using non-parametric Wilcoxon´s signed rank test. Analysis of variance (ANOVA) with Bonferroni's post hoc test was used to compare multiple groups. For all comparisons, a P- value < 0.05 was considered to be statistically significant. All values were expressed as means ± SEM.

Results and Discussion

Islet Morphology and Vasculature in TSP-1 Deficient Mice

The pancreas weight did not differ between wild-type and TSP-1 deficient mice at either 10-12 weeks, 16 weeks, or at one year of age. The islet cell mass was higher in 10-12 week old TSP-1 deficient mice when compared to age-matched wild-type mice, but, at 16 weeks and one year of age no difference in islet cell mass was seen. The fractions of α- and β-cells were similar in both groups of mice and at all ages. Both α- and β-cell masses were increased in 10-12 week-old TSP-1 deficient mice when compared to age-matched wild-type mice. However, this difference was no longer present at 16 weeks of age. No differences in total number of islets, or difference in islet size, were noticed between each age-matched groups. Both groups were found hypervascular at one year of age, where TSP-1 deficient mice were found to have more islet blood vessels than corresponding wild-type animals, likely due to a late and age-related decrease of islet mass. However, there were no differences in islet vascular density at 10-12 weeks or 16 weeks of age between TSP-1 deficient and wild-type mice. Concordantly with this, the blood perfusion of both whole pancreata and the pancreatic islets from 10-12 week-old were similar in wild-type and TSP-1 deficient mice.

When TSP-1 deficient mice were first characterized in 1998, young pups of these mice (<2.5 week of age) had an almost normal morphological phenotype, but for the pancreatic islets [9]. In Paper I we show that the islet vascular changes including hypervascularity and hyperplastic islets in young TSP-1 deficient mice [88] are transient and disappear before 10-12 weeks of age, showing at this latter age, no difference in vascular density and islet blood perfusion compared to control mice. These results could be interpreted as although an early vascularization of islets postnatally, or after transplantation, is facilitated by low TSP-1 levels, and creates a compensatory increased expansion of endocrine cell mass in TSP-1 deficient mice during their first months, the islet vascular density is normalized later on. This is in accordance with our previous findings of a similar situation where an initial increased vascularity and blood perfusion in young obese-hyperglycemic (ob/ob) mice was normalized after islet mass expansion with time [233]. In other settings such as perinatal islet growth in normal rats and during rat

pregnancy, it has been suggested that an improved vascular support might be helpful to stimulate the islet endocrine cell proliferation [22, 234].

TGF-β1 has an important role in pancreatic islet morphogenesis during rat pancreatic development through the regulation of MMP-2 [97]. The results in Paper I suggest that the early postnatal effects of TSP-1 and TGF-β on islet morphology are either transient or compensated for after the first months by other mechanisms. Earlier findings have suggested that TGF-β blocks the mitogenic response of β-cells to glucose [235] and the same study shows that overexpression of TGF-β generates small islet clusters compensated for by an increased numbers of cells to obtain a normal islet cell mass [236]. However, no difference was noted with regard to either islet numbers and islet size distribution between TSP-1 deficient animals and wild-type controls. Treatment of neonatal TSP-1 mice with TGF-β1 activating sequence of TSP-1 prevented not only development of islet morphological changes [9], but also β-cell dysfunction in these mice. However, in Paper I, displaying the follow up of TSP-1 deficient mice after one year, the morphological changes were transient, whereas the functional defects persisted at one year of age (for functional aspects see later section).

Identification of Receptors and Their Localization

Ghrelin mRNA and the receptors for ghrelin have in previous studies been detected in a variety of tissues including blood vessels and heart, suggesting a direct local effect on the vessels [237]. In our study the mRNA expression for the receptor for ghrelin, GHS-R1*α,* was observed both in whole islet and specifically in islet arterioles, where the normalized expression of GHS-R1*α* was six times higher when compared to whole islets. Ghrelin (GHRL) gene expression was only detected in whole islets, not in the arterioles. Immunohistochemical double staining for GHS-R1*α* and glucagon cells showed that GHS-R1*α* receptors are located to the periphery of the islets, with a co-localization to the glucagon-positive *α*-cells. GHS-R1*α* was also partly co-localized to vascular smooth muscle cells at the vascular pole, but not to the CD31 positive micro vascular EC. This indicates that GHS-R1*α* is in the right position in order to exert effect locally in the pancreas on blood perfusion.

In Paper III, our attempt to influence β-cells with CART and an ET-1 receptor antagonist BQ-123 simultaneously did not render an effect, which suggests that CART exerts its effect via other mechanism than ET-1 receptors.

In Paper IV, we observed irisin in α-cells in both mouse and human islets. Irisin was overlapping predominantly with glucagon in immunohistochemistry sections. Previous studies have only located irisin to the islets, without being able to specify the cellular origin [182]. Since glucagon is released at

low glucose levels, and irisin at high glucose levels, the location of irisin is suggested to be in other granules than those containing glucagon, and the mechanism for its release to depend on separate intracellular machinery, something that might be an interesting target for future studies.

For CART and irisin we could not identify any receptor, in concordance with earlier studies. However, the localization of irisin to α-cells is interesting and indicates an endocrine function of the hormone.

The importance to find receptors is pivotal for the full understanding of local endocrine interactions. Despite numerous studies on the physiological effects of CART, its receptor is still unknown. This could be explained as CART being promiscuous in its nature and therefore acts through several different receptors either directly or via mediated effects. If so, this could explain some of the contradictory effects ascribed to CART in different earlier publications. Among previous suggestions and theories, from studies on rat brain and mouse pituitary cells, respectively, CART has been proposed to potentially act through a G protein-coupled receptor mediating inhibition of adenylate cyclase (G- $\alpha_{i/o}$) [238, 239]. CART alters the MAPK-cascade and phosphorylate ERK 1 and 2, and CART signaling could be blocked by pertussis toxin (PTX), indicative of CART acting on a G-protein coupling receptor through a $G_{i/o}$ mechanism with the involvement of upstream kinases, MEK1 and 2 [240]. Another finding is that CART stimulates production of cAMP, which indicates action through a $G_s\alpha$ - coupled receptor [159, 241].

Islet Blood Flow

In paper I, TSP-1 deficient mice showed no difference in either PBF or IBF when compared to wild-type mice.

In paper II, PBF was not affected by either ghrelin or GHRP-6 administration, but the IBF was, selectively, decreased by ghrelin in fed rats. In contrast, in fasted rats, GHRP-6 administration caused a fourfold increase in IBF. The fraction of whole PBF diverted through the islets (IBF %) was similar in saline, ghrelin and GHRP-6 treated fed rats, while in fasted rats there was a marked increase of IBF % following GHRP-6 administration.

In Paper III, CART did not affect splanchic blood flow and the mean arterial blood pressure was maintained between 100-120 mmHg in all animals. The PBF was similar in the control and CART group, with or without simultaneous glucose administration and the PBF was neither affected by administration of BQ-123. At the same time IBF was markedly decreased by CART infusion, and in the setting where glucose administration increased the IBF the simultaneous administration of CART did partly decrease this effect. In the same experiments colonic blood flow (CBF), duodenal blood flow (DBF) and renal blood flow (RBF) were not affected by either CART or BQ-123, however glucose alone increased CBF and DBF.

In Paper IV, irisin decreased IBF by nearly 50 %, while not affecting mean arterial blood pressure, PBF, CBF, DBF, or RBF. However, irisin decreased the blood flow in WAT, without a similar effect on BAT.

Many factors known to increase IBF in response to an increased metabolic demand have been identified in previous studies, for instance glucose [37], tolbutamide [242] and glucagon [10]. However, factors associated with a decreased insulin demand have yielded conflicting results in vascular regulation [243-245]. Insulin administration decreases IBF, but this is likely due to the resulting hypoglycemia, rather than a direct effect of hyperinsulinemia [246]. There are other nutrients and hormones that have indirect effects on IBF by interacting with vasodilators and vasoconstrictors [46, 247], and also the central nervous system impacts islet hormone secretion [248]. All together these summarize that IBF is controlled by multiple factors and that this is connected with the metabolic state of the organism [249].

In Paper II, ghrelin was found to be a factor that decreases IBF. Inhibition of endogenous ghrelin concentrations by GHRP-6 in fasted rats further supported this through a substantially increased IBF. The explanation may be that the increased ghrelin concentration that occur during fasting may act to decrease the blood perfusion of pancreatic islets, this thought to be due to a decreased demand for islet cell activity during states of fasting.

Additionally, CART, in Paper III, did decrease IBF without affecting other regional blood flows. In previous studies, ET-1 has been suggested as an important mediator of the direct constriction of vascular tone by CART [168, 169]. In these publications ET-1 receptor antagonist BQ-123 blocked the vasoconstriction effect of CART in cerebral arteries in rats, but we did not observe any similar effect from the ET-1 receptor antagonist in our experiments on the rat pancreatic vasculature. Therefore the specific mechanism performed by CART remains unknown.

In Paper IV, the finding of the large IBF decreasing effect of irisin was unexpected, since irisin was secreted following higher levels of glucose concentrations. During hyperglycemia there is a demand for increased IBF to match the need of augmented insulin release. The finding could mean that irisin has a role in a negative feedback mechanism in order to prevent capillary damage and hypertension at prolonged hyperglycemia [250]. Another possible explanation is the connection of irisin to exercise. Perhaps is the amount of irisin secreted locally in this setting too low to affect blood flow compared to the amounts generated by exercise. Exercise in itself is associated with a decrease in IBF, consistent with that in an exercise state, where insulin secretion is already inhibited by sympathetic innervation and circulating catecholamines [251]. Therefore, if irisin aids in the decrease of IBF, it conforms well to a decreased demand for insulin secretion.

In general, dynamic interactions between blood vessels and β-cells are important for the islet function [11], a focus of this thesis. Decreased islet vas-

cularization or blood flow can have negative effects on islet insulin secretion and whole-body glucose tolerance. Conversely, increased islet vascularization has the opposite effect whereas dysfunctions in the vasculature show connections to the pathogenesis of T2D, early noticed as changes in skeletal muscle blood flow [249, 252, 253]. So far there is sparse information saying that PBF is affected by T2D, however there are much more evidence that IBF is affected [10]. IBF is affected in T2D both due to vascular morphological changes and endothelial and SMC dysfunction in the islets, and also due to functional changes in the vascular regulation, partly since hyperglycemia stimulates the production of NO and causes decreased endothelial cell survival [254]. Also, specific arteriole dilatation in response to glucose was enhanced in islets derived from diabetic animals [223]. There are several experimental models of T2D or models with impaired insulin tolerance, and a common feature for them is the association with increased IBF [10, 43]. An IBF increase is however only prominent early in the disease, but with time and a diminished number of β-cells, it decreases.

To summarize, T2D, in its early phase, is associated with increased IBF, and vascular dysfunction. Perhaps is the increase in blood perfusion causing an increased vascular shear stress [255-257], the cause for the endothelial and β-cell damage, and thus therapies that would decrease IBF would be beneficial in the treatment of T2D. In this thesis, three of the factors (Paper II-IV) clearly exert this specific function.

Glucose Tolerance and Islet Function *in Vivo* and *in Vitro*

All animal groups in Paper I had normal non-fasting plasma glucose values. However, an ivGTT performed at either 10-12 weeks or in one-year-old TSP-1 deficient mice showed impaired glucose tolerance. Additionally, serum insulin concentrations 10 min after glucose administration were substantially lower in TSP-1 deficient mice when compared to wild-type mice at one year of age. In additional experiments, transplantation of adult TSP-1 deficient islets into wild type recipient animals at 10-12 weeks of age, in order to try to reconstitute TGF- β1, did not affect the β-cell dysfunction.

When challenged with high glucose (16.7 mmol/L) TSP-1 deficient islets implanted to wild-type mice responded in the same way as TSP-1 deficient islets implanted to TSP-1 deficient mice. Since transplanted islets derive most of their new vascular system from the recipient [258-260], this indicates an irreversible β-cell dysfunction induced by persistence of TSP-1 deficiency, if not substituted early in life. The latter situation has been shown in a previous study from our group where neonatal TSP-1 deficient islets regained function after early revascularization by TSP-1 positive blood vessels derived from the recipient [88].

TSP-1 deficiency in islets caused vascular and endocrine morphological alterations postnatally that normalized with time, but the TSP-1 deficient mice had chronic β-cell dysfunction. Therefore, the support of TSP-1 is important for the maintenance of GSIS and thereby normal glucose homeostasis.

In paper II the fasted animals had an increased serum ghrelin concentration, and this was not affected by an ivGTT. The ghrelin receptor inhibitor GHRP-6 had no effect on serum insulin concentrations during an ivGTT in fed rats, and the peak glucose concentration at 15 min after the administration of glucose was similar in GHRP-6 and saline treated animal, but the glucose disposal rate was significantly impaired in the GHRP-6 treated animals. The situation was markedly different in fasted rats where GHRP-6 pretreatment caused an increased serum insulin peak concentration five minutes after glucose administration and a much lower blood glucose peak during the ivGTT when compared to the saline treated animals. In the ipITT, there was no effect on glucose levels regardless of whether ghrelin was blocked or not.

GHRP-6 had no effect on the insulin release from islets obtained either from fed or fasted rats at low glucose concentration (1.67 mmol/L), but when the islets were exposed to a high glucose concentration (16.7 mmol/L), islets irrespective of whether obtained from fed or fasted rats showed an enhanced insulin secretion when exposed to GHRP-6.

The effect of ghrelin on insulin secretion has, historically, been a matter of debate. A majority of studies shows that ghrelin acts as an insulinostatic agent, mediated through GHS-R1α that is expressed in the β-cells [112, 139]. This causes inhibition of insulin release in humans, rats, mice as well as from clonal β-cells [112, 261-264]. However, there are also some previous studies that instead have indicated a stimulatory effect on insulin release [111, 265], and this difference has been thought to reflect methodology, and different experimental settings. These differences where examined in detail by Salehi *et al.* [266] and they found a dose dependent behavior of ghrelin on insulin release in mice showing GSIS suppression at low ghrelin concentrations, and GSIS potentiation at high concentrations. This difference was later explained as ghrelin performing the insulinostatic action on the G α_{i2} subtype of GTP binding proteins and delayed outward K^+ (Kv) channels, which suppress $[Ca^{2+}]_I$ signaling in β –cells [140, 267]. The stimulation of G α_{i2} rather than the G α_q subtype of the GTP binding proteins suppresses the cAMP-signaling pathway in β-cells. This suppression of cAMP in turn, activates Kv channels, thereby suppressing glucose-induced action potentials, $[Ca^{2+}]_i$ increases due to membrane excitability, and consequently suppresses Ca^{2+} influx [139, 268]. This causes inhibition of insulin release in the β-cells [267]. Different previous studies focusing on receptor inhibition, e.g. by inhibiting voltage dependent K^+ channel [140], by blocking the Gi/Go subtypes of GTP-binding proteins [139], or by the presence of cAMP analogues

and inhibitor of adenylate cyclase [269, 270], further support that ghrelin inhibits insulin release primarily via G α_{i2} - mediated inhibition of the cAMP-PKA-pathway, which in turn enhances Kv2.1. channels and suppresses $[Ca^{2+}]_i$ signaling in β –cells [139].

Another theory discusses the formation of heterodimerization between GHS-R1α and the somatostatin receptor, arguing that the ratio between ghrelin and somatostatin is important for the effect on GSIS. When the ratio is in favour for ghrelin, the GHS-R1α proceeds towards G α_{i2} coupling, and the above described process follows, whereas a low ghrelin ratio destabilizes the heteromer conformation, restoring G α_q subtype coupling, resulting in no effect exerted by ghrelin [271]. However this heterodimerization has been difficult to reproduce in other studies. Instead another connection between ghrelin and somatostatin has been demonstrated, where ghrelin promotes somatostatin release through GHS-R1α in the δ cells instead, through subunit G α_q and $[Ca^{2+}]_i$ increase, which would then inhibit insulin release via a negative ratio between ghrelin and somatostatin, where this ratio would direct the GHS-R1α coupling to either the G α_{i2} or G α_q subunit [272, 273].

The different results, in our study (Paper II), *in vivo* and *in vitro* might be due to an accumulation of ghrelin in the tissue in the absence of vascular drainage. A similar situation has been observed for islet amyloid polypeptide (IAPP) in islet culture, or following microencapsulation [274]. The amelioration of GSIS from the pancreas by fasting has been known for decades [275] and with the findings in Paper II it seems that ghrelin is a contributing factor with its attenuation of insulin secretion from the islets. An important part of our study was that ghrelin antagonism increased peak insulin concentrations in fasted rats only. Therefore, it may be that endogenous ghrelin during fasting exerts islet vascular effects to diminish the blood perfusion of the tissue and thereby the insulin release from the islets. Some earlier findings such as pathophysiological up-regulation of angiotensin II in islets [276] and disruption of insulin receptor signaling in islet EC [277] have noted an impaired insulin secretion from islets *in vivo*. With this findings in Paper II, ghrelin is, to our best knowledge, the first factor reported showing physiological restriction of insulin delivery from the pancreatic islets by acting on the vasculature.

Despite significant effects on the IBF, CART did not affect serum insulin concentrations at any time during the ivGTT. Both the insulin values and the glucose values were unaffected by CART when compared to control. Also *in vitro* experiment studying glucose-stimulated insulin release of isolated islets, displayed no difference between CART and the control group neither in a setting with an acute effect from CART, or in a setting where the islets where pre-incubated with CART.

CART may exert different effects on pancreatic islets depending on species [154]. In rats, CART seems to augment GSIS through a cAMP/PKA mediat-

ed pathway, but without the presence of cAMP elevating agents, no stimulation takes place, and in isolated rat islets, naturally without any cAMP-elevating agents, CART is a weak inhibitor of insulin, glucagon and somatostatin [159, 278]. In mice and humans, CART stimulates GSIS, an effect also present *in vitro* in these species [154, 158].

For irisin (Paper IV) islet perifusion shows that irisin is increased in response to glucose. In the same setting, potentiation of insulin release by forskolin did not alter the secretion of irisin regardless of high or low glucose state. *In vitro*, in rat and human islets, at both high and low glucose incubation, irisin or irisin neutralizing antibody had no effect on insulin or glucagon secretion.

Irisin was observed to be secreted in response to increased glucose levels despite irisin being located in α-cells. In our study no effect from irisin was seen on insulin secretion, something that is in contrast with an earlier study where GSIS was augmented after irisin incubation in human and murine islets as well as INS-1E cells [218]. At the moment the difference in our observations is not obvious to explain, since similar doses and methods are used as in our study. Therefore further studies are needed before an exact role of irisin on insulin secretion could be determined.

Conclusions

In the first study we concluded that TSP-1 deficiency in islets causes vascular and endocrine morphological alterations with increased endocrine cell mass and hypervascularity postnatally. The morphological changes are transient and the islet vascular density normalizes before 10-12 weeks of age and onwards. However, for unknown reasons, the islets were again hypervascular in one-year-old rats. β-cell dysfunction was present in all TSP-1 deficient mice, both *in vivo* and *in vitro*. Also, when trying to reconstitute TSP-1 in TSP-1 deficient animals through a transplantation model, adult islets failed to recover. Thus local support of the islet endothelial product TSP-1 is identified as an important factor for maintenance of glucose-stimulated insulin secretion and thereby glucose homeostasis.

In the second study the GSR-1α receptor was identified in both arterioles and in islet endocrine cells, verified both by PCR and immunohistochemistry, whereas ghrelin production was only present in the islets and not in the arterioles. Immunohistochemistry further suggested a co-localization with the α cells. The GSR-1α receptor inhibitor GHRP-6 increased IBF in fasted rats, but with no effect in fed animals. At the same time no effect was seen on pancreatic- or other regional blood flows. GHRP-6 also increased the circulating insulin concentrations after glucose administration to fasted rats *in vivo*, and to all islets *in vitro* when islets were exposed to high glucose concentrations, suggesting a direct effect by GHRP-6 on the islet cells. In summary ghrelin affects IBF during fasting to meet the decreased demands for systemic insulin delivery. By blocking this effect with GHRP-6, acting locally, causing an increased IBF and glucose stimulated insulin secretion, GHRP-6 might be beneficial to improve islet cell function.

In the third study the islet derived factor Cocaine- and amphetamine-regulated transcript (CART) decreased IBF without affecting PBF or systemic blood flow. Simultaneous co-administration of the ET-1 inhibitor BQ-123 had no effect on IBF. Concurrently, no effect was noted by CART *in vivo* in the ivGTT or *in vitro*, on rat islets with regard to insulin release.

In the fourth study the major finding was that irisin was found to be co localized with α- cells. Previous histological studies have only demonstrated the presence of irisin in the endocrine pancreas. Further, irisin specifically decreased IBF without affecting PBF. No effect was observed from irisin on the insulin secretion neither *in vivo* nor *in vitro*, but irisin was secreted in response to increased glucose levels. This is contradictory but could be a mechanism to protect β-cells from glucotoxicity at higher glucose levels by reducing blood flow and thereby the amounts of glucose delivered.

In conclusion, this thesis discusses four different factors, all with a potential to affect islet function. To fully elucidate the physiological role of these, further studies are needed, and for the latter two candidates the identification of a receptor is pivotal.

Sammanfattning på svenska

Typ 1 diabetes är en sjukdom som uppstår då kroppens egna immunceller attackerar (autoimmun reaktion) de så kallade beta-celler vilka finns i de Langerhanska öarna i bukspottskörteln. Det leder till att insulinproduktionen upphör helt. Diabetes finns i två former där man vid den andra formen, typ 2 diabetes, istället har en otillräcklig insulinproduktion i förhållande till behovet. Typ 2 diabetes beror både på sänkt insulinproduktion, det vill säga på ett för litet antal beta celler som producerar insulin och att cellerna dessutom inte förmår att svara som tidigare med ökad insulinproduktion när blodsockret stiger, samt på en generellt försämrad insulinkänslighet ifrån kroppens övriga vävnader, vilket leder till att det krävs en större mängd insulin för att cellerna skall kunna ta emot och upp sockret ifrån blodet. Detta beror på en kombination av ärftlighet och miljöfaktorer, såsom till exempel fetma och övervikt. Typ 1 diabetes behandlas med insulin, emedan typ 2 diabetes behandlas med rådgivning, motion och viktnedgång samt läkemedel som förbättrar insulinkänsligheten och -produktionen. För de svårare typ 2 diabetes fallen krävs dock slutligen också insulintillförsel. Diabetes ger, förutom problem med insulinfrisättning och blodsockerbalansen även följdkomplikationer i form av kärlskador. Dessa uppstår på grund av att de höga sockerkoncentrationerna i blodet (hyperglykemi) retar kärlen och via olika mekanismer därför skadar dem. Detta gestaltar sig både som mikro- och makrovaskulära skador, det vill säga småkärlspåverkan på bukspottskörteln, njurar, ögon, nerver och fötter, samt storkärlspåverkan med risker såsom stroke och hjärtinfarkt. Diabetes kan med andra ord påverka i stort sett alla kroppens organ genom en generell påverkan på blodkärlen.

I bukspottskörteln (pancreas), finns det ungefär 98 % exokrin vävnad och 2 % endokrin vävnad. Det är den endokrina vävnaden som är av intresse vid diabetes. Den endokrina vävnaden utgörs av de Langerhanska öarna, vilka upptäcktes av medicine studerande Paul Langerhans 1869. I de Langerhanska öarna finns förutom redan nämnda beta-celler fyra ytterligare ö-cellstyper som producerar hormon; glukagonproducerande alfa-celler, somatostatinproducerande delta-celler, polypeptidproducerande polypeptidceller samt epsilon-celler som producerar ghrelin. Utöver detta produceras flertalet mindre kända hormoner ifrån bukspottskörteln och några av dessa är i fokus i denna avhandling. Förutom hormoner regleras bukspottkörteln även av nervsystemet och framför allt genom kontakt mellan ö-cellerna och dess blodkärl, och då särskilt endotelcellerna som finns på blodkärlens insida.

Särskilt det sistnämnda har varit centralt för denna experimentella avhandling. Mer specifikt har mitt arbete inriktat sig på lokalt producerade faktorer och deras påverkan på bukspottskörtelns funktion, såsom frisättning av insulin, samt dess blodflödesreglering. En viktig del i detta utgörs av mätningar av det lokala blodflödet och det forskningsområdet är något som vår forskargrupp jobbat mycket med historiskt genom utveckling och modifiering av tekniker kring det. Den centrala tekniken för detta är så kallad mikrosfärteknik, det vill säga att man intravenöst injicerar små plastkulor i försöksdjuret, vilka sedan sprider sig i dess blodcirkulation. Man kan sedan räkna hur dessa sfärer fördelat sig i vävnaderna med hjälp av mikroskop, och genom att jämföra detta med ett referensblodprov med sfärer, tagit samtidigt som sfärerna injicerades, avgöra vävnadsgenomblödningen. Detta har vi främst använt för att jämföra blodflödet mellan endokrin och exokrin bukspottskörtelvävnad, dvs mellan ö-blodflödet och blodflödet i hela bukspottskörteln.

Trombospondin-1

I studie I undersöktes långtidseffekterna i möss av frånvaron av trombospondin-1 (TSP-1), ett glykoprotein som är en naturlig hämmare av nybildandet av blodkärl, så kallad angiogenes. Tidigare studier hade visat att avsaknad av TSP-1 ger förstoring av blodkärl och Langerhanska öar, kombinerat med en nedsatt funktion avseende insulinfrisättningen. Med en långtidsuppföljning på ettåriga möss sågs ingen kvarvarande påverkan på kärl eller öarnas storlek men däremot kvarstod beta-cellernas dysfunktion. Även försök att transplantera isolerade öar från möss med TSP-1 brist till friska möss med TSP-1, i syfte att ersätta deras TSP-1-brist, gav ingen återkomst av funktion. Slutsatsen här är att TSP-1, som producerats lokalt av endotelceller från blodkärlen i ö-cellerna, är en viktig faktor för att upprätthålla god glukosstimulerad insulinfrisättning och på så sätt upprätthålla stabila glukosnivåer.

Ghrelin

I studie II undersöktes inverkan på blodflöde och betacells-funktion av hormonet ghrelin. Ghrelin upptäcktes först i magtarmkanalen men visade sig lite senare även produceras av epsilon-cellerna i bukspottskörteln. Ghrelin ses som ett ”hungerhormon” och ökar i koncentration vid fasta. Som första steg i studie II identifierades receptorer för ghrelin i både arterioler i öarna och i ö-celler, med en co-lokalisation till alfa-celler i de Langerhanska öarna. I studie II konstaterades att genom tillförsel av ghrelin minskas ö-blodflödet selektivt. Genom hämmande av ghrelinreceptorn GSR-1α med Growth Hor-

mone Releasing Peptide-6 (GHRP-6) i fastande råttor kunde istället ö-blodflödet tydligt ökas, men denna effekt saknades helt i icke-fastande djur. Påverkan på ö-blodflödet var samtidigt selektivt då inget annat blodflöde påverkades. GHRP-6 ökade även insulinproduktionen signifikant i fastande djur, men utan påverkan i icke-fastandes, däremot påverkades alla isolerade odlade öar oavsett om de var fastande eller ej. Som slutsats kan konstateras att ghrelin påverkar ö-blodflödet under fasta för att på så vis hantera det minskade behovet för insulinfrisättning. Samtidigt visar en blockering av ghrelin med hjälp av GHRP-6 att både ö-genomblödningen och insulinfrisättningen kan förbättras, varför GHRP-6 kan verka positivt för att förbättra ö-cellsfunktionen.

Cocaine and Amphetamin Regulated Transcript (CART)

CART är en neuropeptid som i studie III använts på liknande sätt som ghrelin användes i studie II. Även här har vi via mikrosfärmätningar kunnat fastställa att CART minskar ö-blodflödet utan att ge samma påverkan på hela bukspottskörtelns blodflöde eller på systemblodtryck. Eftersom CART saknar känd receptor gjordes även ett försök att finna mekanismen till CARTs funktion genom samtidig tillförsel av endotelin-1 hämmaren BQ-123. Endotelin-1 (ET-1) är en känd potent blodkärlssammandragande faktor, som produceras av endotel, och en tidigare studie hade ingett misstanke om att CART kunde interagera med ET-1, men vi fann ingen sådan koppling i vår studie. CART hade inte heller någon effekt på bukspottskörtelns insulinfrisättning, varken vid ett glukostoleranstest (där socker injiceras i blodet i syfte att testa hur bukspottskörteln svarar följt av att man mäter blodsockernivåer och insulinnivåer vid förbestämda tidpunkter) i råtta eller vid en liknande test på isolerade rått-öar. CART har således en selektiv påverkan på ö-blodflödet, men utan att påverka beta-cellernas funktion, något som skulle kunna innebära att CART försöker att skydda ö-cellerna mot den kärlpåverkan som höga glukosnivåer kan ge.

Irisin

Även studie IV fokuserade på blodflödesmätningar där effekten från irisin, ett myokin, dvs en peptid producerad och frisatt från muskel, utvärderades. Som första fynd noterades irisin i alfa-celler, något som är nytt då förekomsten av irisin tidigare endast konstaterats ospecifikt till ö-cellerna. Irisin påverkade ö-blodflödet utan påverkan på hela bukspottskörtelns blodflöde, eller blodflödet i övrig i undersökt vävnad. Irisin hade ingen påverkan på

insulinfrisättningen men däremot frisattes irisin som svar på höga glukosnivåer. Detta är en aning svårtolkat men kan ses som en mekanism för att skydda beta-cellerna från den lokala blodsockerförgiftning som annars skulle kunna drabba framför allt blodkärlen vid för höga blodsockernivåer. Irisin förefaller med andra ord verka blodkärlsskyddande.

Slutsats

Den långsiktiga möjligheten med ökad kunskap kring lokalt blodflöde i bukspottskörteln är att finna verktyg för att påverka dessa faktorer, något som skulle kunna användas för att optimera beta-cellernas funktion och kanske främst användas som komplement tillsammans med andra behandlingsmetoder. Behandlingen kan även bli aktuell för en generell optimering av grundfunktionen vid pre-diabetes, men även för att understödja den eventuella funktion som finns kvar efter att sjukdomen brutit ut. I denna avhandling har fyra faktorer undersökts och utvärderats, alla med potential att påverka bukspottskörtelns endokrina funktion. För att utföra ytterligare kartläggning av den fysiologiska funktionen för dessa faktorer krävs mer studier och avseende de två sistnämnda blir studier med målet att hitta deras receptorer av extra vikt och intresse.

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