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STUDIES OF PANCREATIC ISLET MICROCIRCULATION AND INSULIN SECRETION IN NORMAL AND DIABETIC RATS

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To my family

ABSTRACT

Type 2 diabetes is increasing globally. The disease is characterized not only by hyperglycemia, but also by insulin resistance with attendant dyslipidemia, hypertension and endothelial dysfunction. These aberrations may result in macrovascular disease, and deeply impact the longevity and quality of life in diabetic patients. Gender differences also exist with more pronounced negative effects of diabetes on the lipid profile and blood pressure in women compared with men. To well control all of these risk factors, diabetic patients frequently need to be treated with a lipid lowering statin, and an ACE inhibitor or angiotensin receptor antagonist against hypertension and/or albuminuria. Such drugs reportedly also decrease the risk of incident diabetes in clinical trials, by unknown mechanisms. The aim of the present work was to characterize the effects of vasoactive drugs, free fatty acids and ethanol on islet microcirculation, *in vivo* insulin secretion and glucose tolerance in normal and diabetic rats of both genders. To this end, a microsphere technique was evaluated and applied for measurements of islet blood flow in rats. Vasoactive drugs that are frequently given to diabetic patients were found to increase islet blood flow (IBF), augment insulin secretion and improve glycemia. This suggests that a local pancreatic RAS plays a substantial role in the regulation of islet microcirculation, thereby impacting insulin secretion and glucose tolerance. Qualitative and quantitative gender-specific differences in responses to such drugs were noted, which may be due to the vasoactive features of sex hormones or other influences conferred by gender. Free fatty acids lowered IBF and may thus worsen hyperglycemia by limiting the supply of insulin needed to curb hyperglycemia. Alternatively, the decreased IBF could represent a protective mechanism by which islet exposure to free fatty acid toxicity can be limited. Ethanol acutely exerted substantial influences on pancreatic microcirculation by

evoking a massive redistribution of pancreatic blood flow from the exocrine into the endocrine part via mechanisms mediated by nitric oxide and vagal stimuli, augmenting late phase insulin secretion, and thereby evoking hypoglycemia. This effect may in part underlie the well known hypoglycemic properties of alcohol in diabetic patients or in alcoholics with hepatic failure. Collectively, these direct islet effects of RAS-interfering drugs, statins, fatty acids and ethanol may prove valuable in designing different and gender-specific treatment strategies for diabetic patients or subjects at risk of developing glucose intolerance. Moreover, vasoactive drugs that are frequently given to diabetic patients may confer additional treatment benefits beyond their systemic effects by increasing islet blood flow, augmenting insulin secretion and improving glycemia.

LIST OF PUBLICATIONS

I. **Huang Z**, Jansson L, Sjöholm Å.
Pancreatic islet blood flow is selectively enhanced by captopril, irbesartan and pravastatin, and suppressed by palmitate.
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IV. **Huang Z**, Jansson L, Sjöholm Å.
Gender-specific regulation of pancreatic islet blood flow, insulin secretion, and glucose tolerance in spontaneously diabetic Goto-Kakizaki rats
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CONTENTS

1 INTRODUCTION 1
1.1 Diabetes mellitus 2
1.2 Pancreatic islets 3
1.3 Microcirculation of the islets 5
1.4 Renin-angiotensin system 9
2 AIMS 13
3 MATERIALS AND METHODS 14
3.1 Animals and drugs 14
3.2 Measurements of islet blood flow with the microsphere technique 14
3.3 IPGTT (intraperitoneal glucose tolerance test) 18
3.4 Blood glucose and serum insulin concentration determinations 18
3.5 Statistical analysis 18
4 RESULTS AND DISCUSSION 20
4.1 Pancreatic islet blood flow is regulated by RAS, pravastatin and free fatty acids in male (I) and female (II) non-diabetic Wistar rats 20
4.2 Pancreatic islet blood flow is enhanced by ethanol (III) 23
4.3 Gender-specific regulation of pancreatic islet blood flow by RAS, pravastatin, and free fatty acids in male and female diabetic GK rats (IV) 25
5 CONCLUSIONS 29
6 ACKNOWLEDGMENTS 30
7 REFERENCES 34

LIST OF ABBREVIATIONS

ABF	Adrenal blood flow
ACE	Angiotensin converting enzyme
Ang I	Angiotensin I
Ang II	Angiotensin II
AT1	Angiotensin II type 1 receptor
AT2	Angiotensin II type 2 receptor
eNOS	Endothelial nitric oxide synthase
HGP	Hepatic glucose production
IPGTT	Intraperitoneal glucose tolerance test
IBF	Islet blood flow
KBF	Kidney blood flow
L-NAME	*N*-ω–nitro-L-arginine methylester
NO	Nitric oxide
PBF	Pancreatic blood flow
RAS	Renin angiotensin system

1 INTRODUCTION

The pancreas is a unique organ, comprising both endocrine and exocrine cells. The endocrine cells are grouped in the islets of Langerhans, which were discovered in 1869 by the German pathological anatomist Paul Langerhans (1). The islets of Langerhans are dispersed into millions of microorgans scattered among the 100-fold more abundant exocrine tissue. Insulin is a natural peptide hormone made by the pancreas and whose major function is to control tissue uptake and storage of glucose (2). In general, both insulin resistance and impaired insulin secretion are required for manifest type 2 diabetes to occur. However, the mechanisms involved in islet dysfunction remain elusive.

The endogenous islets are richly irrigated, *i.e.* they have a well-developed vascular network in order to supply oxygen and nutrients to the islets. Notably, capillaries feeding the islets have highly fenestrated endothelium and blood flow is high compared to exocrine pancreas, thus allowing for rapid and efficient delivery of nutrients to islets. These capillaries, resembling a glomerulus, course through the islet in a tortuous manner that is ideal for blood-cell and cell-blood interactions. In addition, the blood flow to the islets has been found to be disproportionally large (~ 10 % of the pancreatic blood flow for the 1–2 % of pancreatic volume). These features create a favorable environment for delivery of substances from the blood to the islet cells. To what extent alterations in pancreatic islet microcirculation contribute to the development of the disturbed glucose homeostasis and insulin secretion in diabetes is unknown at present. This thesis studied the microcirculation of pancreatic islets and insulin secretion in normal and diabetic rats.

1.1 DIABETES MELLITUS

Diabetes mellitus is a chronic metabolic disorder characterized by hyperglycemia and other metabolic derangements. The World Health Organization recognizes three main form of diabetes: type 1, type 2, and gestational diabetes, which have similar signs, symptoms, and consequences, but different causes and population distributions (3). Basically, all forms are due to the β-cells of the pancreas being unable to produce sufficient insulin to prevent hyperglycemia. Type 1 is usually due to autoimmune destruction of the pancreatic β-cells. Type 2 is characterized by tissue-wide insulin resistance and varies widely; it sometimes progresses to loss of β-cell function and/or mass. Gestational diabetes is similar to type 2 diabetes, in that it involves insulin resistance in women genetically predisposed to developing this condition.

Type 1 and type 2 diabetes are both incurable chronic conditions, but have been treatable since insulin became medically available in 1921 (4), and today usually are managed with a combination of dietary treatment, tablets (in type 2) and, frequently insulin replacement therapy. Gestational diabetes typically resolves with delivery.

Type 2 diabetes causes many complications. Acute complications (hypoglycemia, ketoacidosis or nonketotic hyperosmolar coma) may occur if the disease is not adequately controlled. Serious long-term angiopathic complications include cardiovascular disease (doubled risk), chronic renal failure (diabetic nephropathy remains the main cause of dialysis in developed world adults), retinal damage (which can lead to blindness and is the most significant cause of adult blindness in non-elderly in the developed world), nerve damage (of several kinds), and other vascular damage that may cause poor healing of wounds, particularly of the feet, can lead to gangrene

which can require amputation - the leading cause of non-traumatic amputation in adults in developed world. The major risk factors contributing to the excess of those complications caused by type 2 diabetes include: hyperglycemia, insulin resistance, dyslipidemia, hypertension, smoking, albuminuria, and the procoagulant state (5). Due to the differences in pathogenesis, the treatment strategies also need to be varied. In terms of quantity, the most important complications of type 2 diabetes are macroangiopathies, *i.e.* myocardial infarction and stroke, which cause some 70 % of the deaths related to type 2 diabetes. In contrast to microangiopathies (*e.g.* nephropathy and retinopathy), where the causal relation to hyperglycemia is well supported, the link between hyperglycemia and macroangiopathy is uncertain, at least in terms of the possibility of reducing macrovascular morbidity solely by reducing hyperglycemia. Patients with type 2 diabetes are thus often treated with a lipid lowering statin and an ACE inhibitor or angiotensin receptor antagonist against hypertension and albuminuria in order to achieve adequate reduction of macrovascular risk.

1.2 PANCREATIC ISLETS

The islets of Langerhans constitute approximately 1-2 % of the mass of the pancreas and they are scattered throughout the entire exocrine parenchyma (6). There are about one million islets in a healthy adult human pancreas, with a total weight of ~ 1-1.5 grams (7, 8), whereas in rats there are 3000-4000 islets in the pancreas (9). Even though the number of pancreatic islets is species dependent, their size is fairly constant, ranging between 25-300 μm in diameter. In rodents, each islet contains approximately 2000-4000 cells. Hormones produced in the islets of Langerhans are secreted directly into the blood flow by (at least) four different types of cells: β-cells producing insulin and amylin (65-80 % of the islet cells); α-cells releasing glucagon (15-20 %); δ-cells

producing somatastatin (3-10 %); PP-cells containing pancreatic polypeptide (1 %); ε-cells containing ghrelin (7). Islets can influence each other through paracrine and autocrine communication, and β-cells are coupled electrically to adjacent β-cells (but not to other cell types).

The function of the islet endocrine cells is to produce hormones that regulate especially carbohydrate metabolism. The β-cells mainly produce insulin, which lowers glycemia by suppression of hepatic gluconeogenesis and promotion of glucose uptake and storage in for instance skeletal muscle (10). The α-cells produce glucagon, which increases glucose concentrations by promoting glycogen breakdown in the liver, and thereby protect the body against hypoglycemia. The δ-cells produce somatostatin, which is an inhibitor of the release of other hormones, including insulin and glucagon. The product of PP-cells is mainly released through vagal mediation, and seems to inhibit the exocrine secretions of the pancreas (11, 12). The ε-cells in the islets of Langerhans produce the satiety hormone ghrelin, which recently has been found to regulate glucose-induced insulin release (13).

The mammalian islet has a nonrandom distribution of endocrine cells usually with only β-cells in the center surrounded by a discontinuous mantle of non β-cells (α-, δ-, and PP-cells) (14, 15) **(Fig. 1)**.

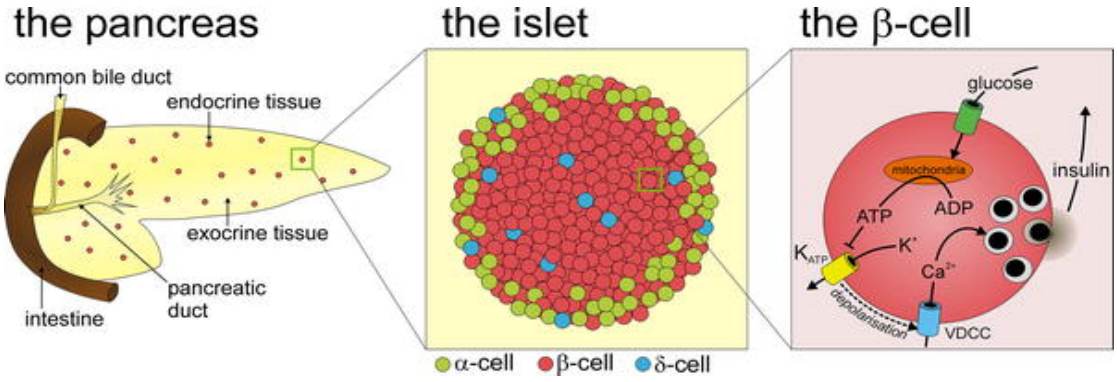

Fig. 1: ***Distribution of endocrine cells in the pancreas***

Human islets have a more complex pattern but can be considered composites of several mantle-core subunits or being lobulated with mantle-core lobules (14, 16, 17). Interestingly, in horse this pattern has been reported to be inverted with a central core of α-cells (18).

1.3 MICROCIRCULATION OF THE ISLETS

The pancreas is part of the splanchnic circulation. The head of the pancreas, which has pancreatic polypeptide-rich and glucagon-poor islets, is supplied by the superior mesenteric artery, while the body and tail, which have glucagon-rich and pancreatic polypeptide-poor islets, are supplied by the celiac artery via the splenic artery (19, 20). The interlobular arteries and vein run in parallel and in close proximity to the major ducts. Thereafter, the intralobular arteries pass straight through the center of the lobuli with branches to islets, acini, and ductuli. In some species the intralobular vein is more peripheral and not parallel to the artery (19, 20), whereas in rats the intralobular vessels run parallel to each other (21).

The anatomic microvasculature of the pancreatic islets has been extensively studied by dye injection, vascular corrosion casts, *in vivo* microscopy and non-radioactive microsphere techniques (6). In these studies, the islets were found to be substantially more vascularized than the exocrine tissue and to be filled first with infusion, and actually have a direct arteriolar blood flow as well **(Fig. 2)**.

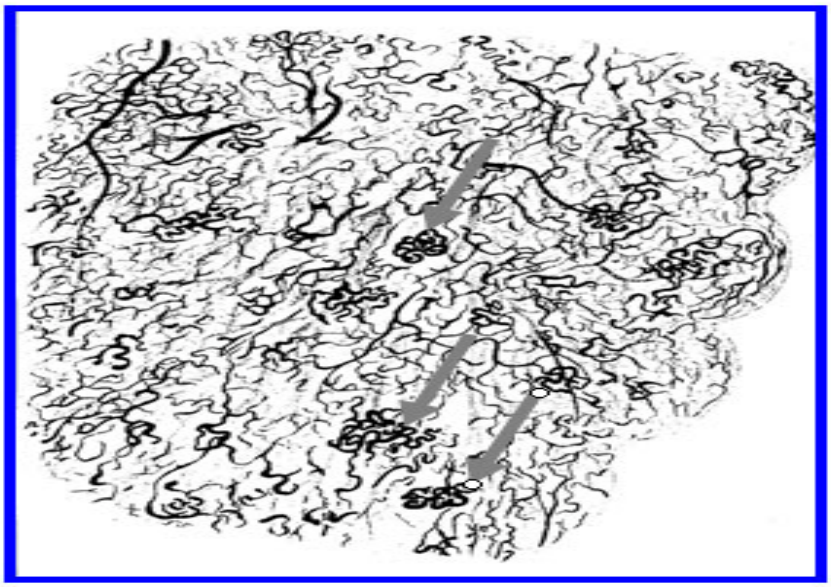

Fig. 2: ***Ink injections showing pancreatic vasculature***

Adopted from Kühne and Lea, 1888.

Rodents have a well-developed insulo-acinar portal system that is similar to humans (22, 23). In rodents, the flow pattern is from centre towards to the periphery (24). The microvasculature pattern may vary depending on the size of the islet in the rat (21). Small islets (< 160 μm in diameter) usually have one arteriole and empty into several small efferent venules that are either connected to exocrine capillary plexa, thereby forming an insulo-acinar portal system, or empty into large veins in the exocrine parenchyma. Islets exceeding 160 μm in diameter receive an arterial blood supply from one to three arterioles and the efferent collecting venules drain into large veins without forming any insulo-acinar system (6) **(Fig. 3)**.

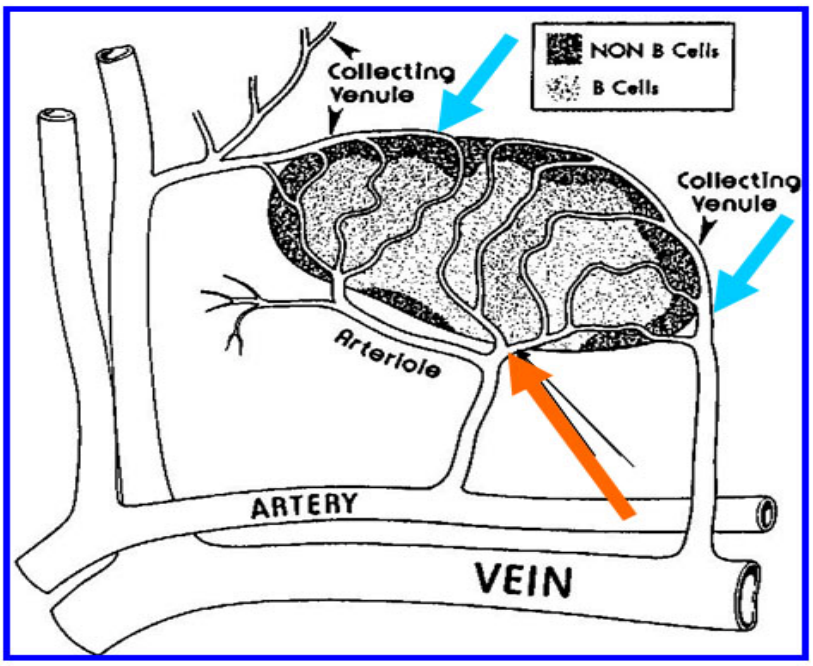

Fig. 3: ***Vascular organization in a large islet***

Adopted from Bonner-Weir and Orci, Diabetes 31:883, 1982.

The islet capillaries have been shown to be 10 times more fenestrated than the capillaries in exocrine parenchyma (25). These fenestrations and high vascular density are dependent on vascular endothelial growth factor (VEGF), which is produced by the islet cells (26, 27).

Since the cell types have specific locations within the islet, the pattern of blood flow through the islet has been proposed to have a significant impact on the ability of cells to intercommunicate within the islet **(Fig. 4)**.

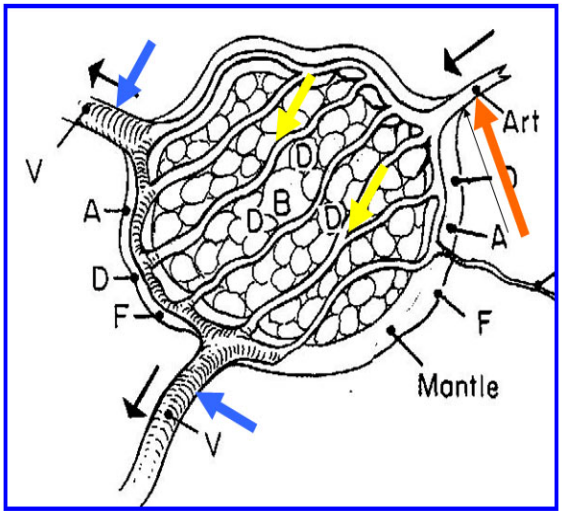

Fig. 4: ***Islet blood flow regulatory sites***

Brunicardi et al, Diabetes 45:385, 1996

This has given rise to three competing models of islet microcirculation. The first model, using scanning electron microscopy of corrosion casts, describes that non β-cells are perfused before β-cells (19, 28, 29). The second model is based on morphological and physiological studies using corrosion casts or anterograde and retrograde pancreas perfusions with anti-insulin/anti-somatostatin gammaglobulin (30). These studies suggest that β-cells are perfused before non β-cells in both rodents (21, 30) and humans (31). Model three describes a gated portal pattern from the afferent to efferent pole of the islet based on intravital microscopical studies (32-34).

Non-radioactive microsphere techniques allow a continuous study of the islet blood flow during prolonged time periods, and have been extensively used to study islet blood flow in experimental animals (35). In rats, the islets have been shown to receive 5-10 % of the total pancreatic blood flow (8, 9, 36, 37), 2-5 % in mice (38) and 15-20 % in rabbits (39), even though they only compose 1-2 % of the total pancreatic volume. The small islets receive only a minor part of the total islet blood flow within the pancreas (9), and then empty out the exocrine capillary system (6). This means that the

flow to these islets is regulated by mechanisms common to those of the whole pancreatic gland. Because of the small fraction of the whole islet blood flow which is diverted to these islets, they are probably of minor importance for whole pancreatic islet blood flow. The larger islets, which receive the major part of the blood flow (9), are supplied by one or three afferent arterioles each. Transplanted isolated pancreatic islets show high basal blood flow values similar to that of the islets within the normal pancreas (40-42). This means that islet blood flow can be regulated separately from the flow to the whole pancreas. An appropriate blood perfusion is essential for maintenance of islet metabolism and insulin secretion. Normally, the blood flow to endogenous islets is tightly regulated at the arteriolar level by nervous, endocrine and metabolic mechanisms (35, 43). Islet blood flow can be preferentially increased from ~ 10 % in a dose-dependent and time-dependent manner after glucose administration (44). Nonetheless, this increased blood perfusion seems to appear only in those islets that have the highest basal blood perfusion (45, 46). The vagus nerve is the most important mediator of this increase in islet blood flow, which is influenced by glucose sensing mechanisms in the brain, oral cavity, and duodenum (47, 48). Furthermore, similar glucose sensors are present in the liver; however, their regulation of islet blood flow is mediated by sympathetic nerves (49). Islet blood flow can be also regulated by local substrates, most notable are adenosine (50) and nitric oxide (NO) (51). Recent research has also shown that angiotensin II (Ang II) is a potent vasoconstrictor in the endocrine pancreas (52).

1.4 RENIN-ANGIOTENSIN SYSTEM

The renin-angiotensin system (RAS) is a peptidergic system with endocrine characteristics. It is classically known as a circulating or hormonal system regulating

whole body blood pressure, electrolyte, and fluid homeostasis (53). The endocrine function of RAS is mediated largely by its potent effects on vascular smooth muscle and on the renal reabsorption of electrolytes as well as water via direct tubule actions, and via stimulation of aldosterone and vasopressin (54). The substrate of the system, angiotensinogen, an α-glycoprotein, is released from the liver and is cleaved in the circulation by the enzyme renin and secreted from the juxtaglomerular apparatus of the kidney to form the decapeptide angiotensin I (Ang I). Ang I is then activated to the octapeptide Ang II by angiotensin converting enzyme (ACE), a key component of RAS, an ubiquitous enzyme that is predominantly expressed in high concentrations on the surface of endothelial cells in the circulation. Ang II, considered the main effector peptide of the RAS, is then acting on specific receptors, for example to induce vasoconstriction by interacting with angiotensin receptors on vascular smooth muscle cells or by stimulating the release of aldosterone from the adrenal cortex (55-57). The biological actions of Ang II are mediated by the angiotensin II type 1 (AT1) and type 2 (AT2) receptors with most of the functions mediated by the AT1 receptor (58) **(Fig. 5)**.

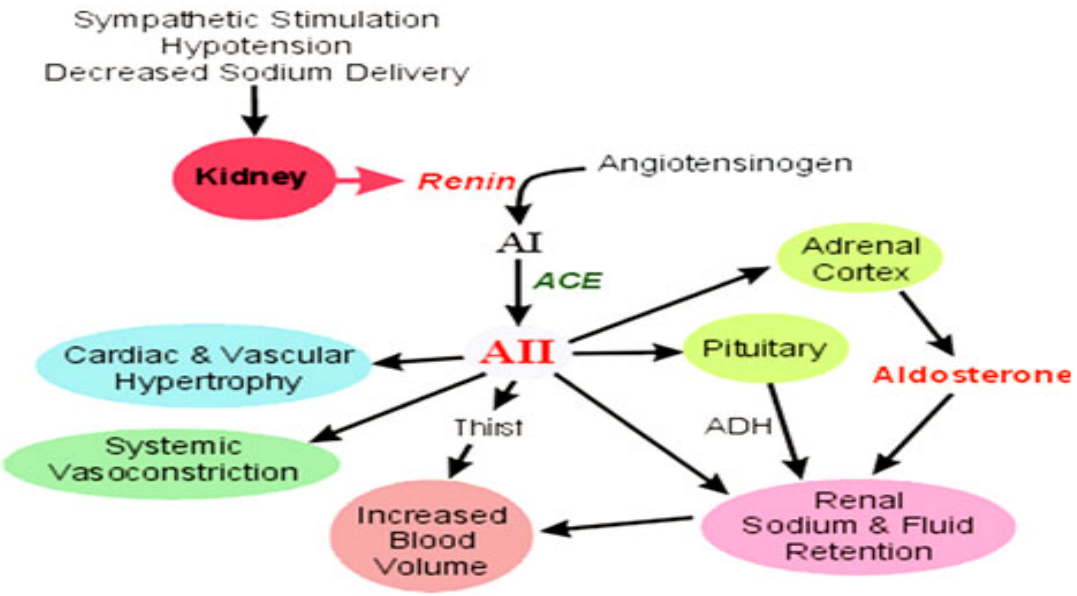

Fig 5: ***A summary of the potential role of the systemic RAS***

The existence of local RASs has been increasingly appreciated over the last 15 years, with mounting recognition of their clinical importance (59). In the pancreas of several species, mRNA encoding angiotensinogen and renin, as well as substantial levels of Ang II, has been detected (60-64). Binding sites for Ang II have been characterized in the endocrine and exocrine cells of pancreas (65-67). Additionally, AT1 and AT2 receptors and Ang II have been specifically localized to different cell types of the pancreas including endothelial, ductal, acinar and islet cells (68, 69). The presence of a local RAS in the human pancreas is further substantiated by the expression and localization of angiotensinogen and AT1 receptors noted in pancreatic islets and ducts (70).

In the endocrine pancreas, islet RAS plays a crucial role in regulating islet glucose-stimulated insulin secretion and thus glucose homeostasis. Ang II has been shown to adversely influence pancreatic and islet blood flow through vasoconstrictive effects (52, 71). Also, high affinity binding sites for Ang II are localized specifically to islet β-cells by double immunostaining and Ang II was found to block glucose-stimulated insulin secretion, an event fully reversible by losartan (an Ang II receptor antagonist) (61).

Accumulating clinical evidence has indicated that pharmacological RAS blockade reduces the incidence of new-onset type 2 diabetes cases in high-risk patients with cardiovascular disease. The mean risk for developing type 2 diabetes was reduced by 27 % with ACE inhibitor treatment, 23 % with AT1 receptor blocker treatment, and 25 % overall in a pooled analysis of these two types of RAS blockers (72). One trivial explanation for this improvement in glycemic control could be that blood flow through the microvasculature to insulin-sensitive tissues (*e.g.* skeletal muscle) increases after treatment with vasoactive substances, thus allowing more insulin to reach metabolically

active tissue. However, the mechanisms for the diabetes preventive effect of RAS blockade still remain elusive and may conceivably also involve direct islet effects.

2 AIMS

The specific aims of the study were:

- to apply a simple method based on the microsphere technique for separate measurements of the blood flow to the endocrine and exocrine parts of the pancreas of the rat.
- to measure alterations in islet blood flow in response to vasoactive drugs currently used in the clinical management of diabetes and to compare these effects between male and female rats and between normal or type 2 diabetic animals.
- to correlate such changes in islet blood flow to changes in insulin secretion and glycemia.
- to evaluate the role of the local pancreatic RAS in islet microcirculation, regulation of *in vivo* insulin secretion and control of glycemia.
- to investigate the impact of ethanol administration on islet blood flow, insulin secretion and glycemia, and to expose the underlying mechanism conveying these actions.

3 MATERIALS AND METHODS

3.1 ANIMALS AND DRUGS

Male and female non-diabetic Wistar rats (ScanBur, Sollentuna, Sweden), weighing approximately 300-350 g, were used in all experiments in paper I, II, III. Diabetic Goto-Kakizaki (GK) rats of both male (300-350 g) and female (200-250 g) gender were obtained from our own local breeding colony (Animal Department, Stockholm South Hospital, Stockholm, Sweden) and used for the experiments in paper IV. All animals had free access to pelleted food and tap water before being anesthetized (Inactin™; Research Biochemicals International; 120 mg/kg of body weight). All experiments were approved by the local ethical committee. Pravastatin and captopril were kindly given by Bristol Myers Squibb. Irbesartan was generously given by Sanofi-Synthelabo. Candesartan was graciously donated by Astra-Zeneca, whereas sodium palmitate was bought from Sigma-Aldrich. All test substances were dissolved in saline except palmitate, which was dissolved in 10 % ethanol solution. Saline was used as a control in all experiments, except when palmitate effects were studied (in which case 10 % ethanol was used as vehicle control).

3.2 MEASUREMENTS OF ISLET BLOOD FLOW WITH THE MICROSPHERE TECHNIQUE

The animals were anesthetized with an intraperitoneal injection of Inactin about 10 min before surgery and placed on a heated operating table to maintain body temperature. A thermistor temperature probe was inserted into the rectum and the body temperature recorded throughout the experiments. The left femoral vein and artery were freed from the surrounding tissue by blunt dissection. A polyethylene catheter with an inner

diameter of 0.58 mm (drawn to a diameter of about 0.35 mm) was inserted into the artery and advanced approximately 1 cm **(Fig. 6)**.

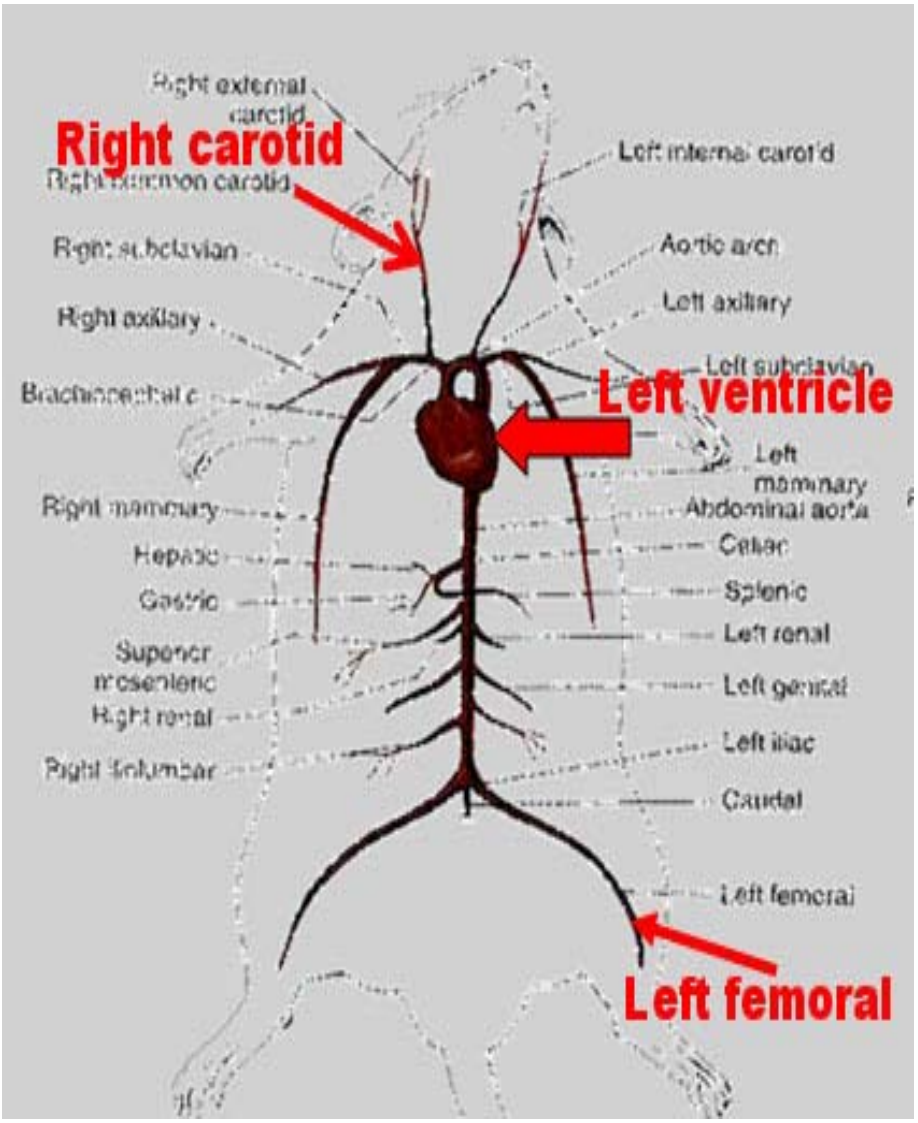

Fig 6: ***Anatomical description of rat arterial branches***

An incision was made on the right side of the neck and about 2 cm of the right carotid artery was carefully freed from its surrounding tissue. Special care was taken to avoid injury to the vagus nerve and the sympathetic trunk. A catheter was inserted into the right carotid artery and is advanced towards the heart (about 4 cm). As the catheter was advanced, a slight resistance was felt when the catheter tip reached the aortic root just prior to entering into the left ventricle **(Fig. 6)**. Care must be taken at this point to avoid perforating the aortic wall. When the tip of the catheter was successfully located in the

left ventricle, the entire catheter exhibited rhythmic movements corresponding to the beating of the heart. If the placement of the catheter in the heart remains unsuccessful, the catheter tip may be left at the aortic root. After making sure it was located in the correct position, the catheter in the right carotid artery was connected to a pressure transducer (PDCR 75/1, Druck Ltd, Groby, Leicestershrie, UK) in order to constantly monitor the mean arterial blood pressure.

When the above procedures had been completed, usually within 15 min, the animals were allowed to equilibrate for 20 min, during which time the blood pressure becomes stabilized. Since the risk of respiratory acidosis increases with time under anesthesia, efforts were made to minimize the time necessary for the surgical procedures prior to the injection of the microspheres.

The animal was then injected intravenously with 1 ml of test substance and rested for another 10 min. During the last minute of the procedure, the preparation for the microsphere injection was done. Nonradioactive microspheres, with a diameter of approximately 10 μm (IMT, Stason Labs, Irvine, CA), were suspended in saline (0.9 % NaCl [w/v]) containing 0.002 % of Tween 80. The microspheres were uniformly suspended by vigorous shaking with the aid of a whirl mixer and 0.5 ml of the suspension (containing between 1.2-1.5 x 10^5 microspheres as determined by counting in a hemocytometer) was injected via the carotid catheter during 10 s. Starting 5 s before microsphere injection and continuing for a total 60 s, an arterial blood reference sample was collected from the catheter in the femoral artery. Additional arterial blood samples were obtained later for analyses of blood glucose and serum insulin concentrations. The polyethylene tube containing the reference sample was weighed to ascertain the exact volume of the sample that must be known to calculate the blood

flow. To prevent clotting, ~ 0.1 ml heparin was added to the blood, which was stored at 4 ^{o}C pending counting of its microsphere content.

The animal was then killed by cervical dislocation, the whole pancreas and both sides of adrenal glands, as well as a 100 mg slice of the kidney (including both cortex and medulla), were removed, blotted and weighed. The pancreas was cut into pieces, each about 5 mm in diameter, which were placed onto microscope slides with about six pieces on each slide. A small piece of adhesive tape (thickness approximately 50 μm) was applied to each end of the slides and the preparation was covered with another slide. The slides were pressed firmly together and held by further strips of tape at both ends, resulting in flattening of pancreatic pieces in the space between the slides. The slides, usually 11 per animal, were frozen and stored at -20 ^{o}C. Counting microspheres was performed immediately after the preparations were thawed and reached room temperature. For this purpose, the pancreas was viewed in a Wild M3 stereo microscope (Wild Heerbrug Ltd, Heerbrug, Switzerland) equipped with both bright-field and dark-field illumination. When the frozen/thawed pancreas was viewed under dark field, each islet stood out as a distinct white spot against the translucent acinar part of the gland.

The number of microspheres present in the reference sample was determined by distributing 40-50 μl portions of the sample on glass microfiber filters (GF/A 2.5 cm, Whatman Ltd, London, UK) with a pore size less than 10 μm. Each filter was placed on a glass microscope slide and counted under bright-field illumination in the stereo microscope (magnification 40X).

If the total number of microspheres in the organ is known, the blood flow to that organ can be calculated according to the formula: $Q_{org} = Q_{ref} \times N_{org}/N_{ref}$, where Q_{org} is organ blood flow (ml/min), Q_{ref} is withdrawal rate of the reference sample (ml/min), N_{org} is the number of microspheres in the organ and N_{ref} is the number of microspheres in the reference sample. A less than 10 % difference in microsphere content between the two adrenal glands was taken to confirm an even distribution of the microspheres in the arterial blood stream.

3.3 IPGTT (INTRAPERITONEAL GLUCOSE TOLERANCE TEST)

The intraperitoneal injection of D-glucose solution (30 % [w/v], 2 g/kg of body weight) was performed in paper II-III. Blood samples were collected from the tail vein immediately before and at 10, 30, 60, 120 min after glucose administration for measurements of glucose and insulin levels. Area under the curve for the IPGTT was determined by computerized image analysis.

3.4 BLOOD GLUCOSE AND SERUM INSULIN CONCENTRATION DETERMINATIONS

The blood glucose concentration was analyzed with test trips (Medisense; Solna), and serum insulin concentrations were measured with an ELISA kit (rat insulin ELISA; Mercodia; Uppsala).

3.5 STATISTICAL ANALYSIS

Values are given as the mean ± SEM. Probabilities of chance differences between experimental groups were analyzed with one-way ANOVA in combination with Student's two-tailed unpaired *t* test. $P < 0.05$ was considered statistically significant.

4 RESULTS AND DISCUSSION

4.1 PANCREATIC ISLET BLOOD FLOW IS REGULATED BY RAS, PRAVASTATIN AND FREE FATTY ACIDS IN MALE (I) AND FEMALE (II) NON-DIABETIC WISTAR RATS

Intravenously injected irbesartan, an AT1 receptor antagonist, or captopril, an ACE inhibitor, induced a robust increase in blood flow not only to the whole pancreas (PBF) and pancreatic islets (IBF), but also in the kidney in both male (paper I) and female (paper II) Wistar rats. Pancreatic blood flow was preferentially redistributed to the islets by both of these agents, an effect that was associated with a significantly increased serum insulin concentration (paper I). Glucose tolerance tests were performed in female Wistar rats (paper II). During the IPGTT, late-phase insulin secretion was substantially augmented and glucose tolerance was significantly improved by captopril treatment. Separate calculations of the total amount of insulin secreted within the 120 min IPGTT (area under the curve) showed that insulin secretion was significantly higher in female rats treated with captopril than in control female rats receiving solvent only. This can be explained by a local or tissue RAS operative in the pancreas (73). During the past few years, it has become increasingly clear that such a local RAS exists in various tissues, implying that local Ang II concentrations could be much higher than those encountered in peripheral blood (60-64). Additionally, Ang II has been shown to adversely influence PBF and IBF through vasocontractile effects (52, 71). It is thus conceivable that pancreatic Ang II, locally produced by intrinsic RAS, may adversely influence insulin secretion *in vivo*, either directly by suppressing β-cell insulin exocytosis or indirectly by limiting islet blood perfusion (52, 61, 71). Evidence also suggests that irbesartan has the capacity to increase vascular dilatation (61, 74) and some publications show that

islet blood flow seems to be suppressed by locally produced Ang II (61, 74), lending support to our present findings.

Dyslipidemia is a salient feature in type 2 diabetes and is known to impair endothelium-dependent vasodilatation (75). The impaired endothelial function may subsequently result in diminished capillary recruitment (76). Pravastatin exerts beneficial effects on endothelial function (77, 78), and may significantly improve selective tissue perfusion by restoring endothelial function. In male Wistar rats (paper I), it was noted a preferential increase in IBF by pravastatin treatment, but no significant changes in PBF, renal (KBF) or adrenal blood flow (ABF). Neither was serum insulin nor blood glucose concentrations affected. Beyond simply lowering cholesterol, the beneficial antithrombotic effect of statins by inhibiting platelet aggregation and promoting local nitric oxide (NO) synthesis (51, 79) may play a role in enhancing endothelium-dependent vasodilatation (75). Since islet microcirculation is extremely sensitive to NO, a local increase in NO production would be expected to preferentially increase IBF, rather than total pancreatic blood perfusion (51). Hence, it is possible, although not proven, that local NO formation might be involved in the advantageous effects of pravastatin noted. Notably, pravastatin appears to be in a class of its own in terms of preventing diabetes (80-82). It is not metabolized by hepatic CYP-450 enzymes, shows very little binding to proteins, and is markedly hydrophilic. Whether these characteristics or other attributes, such as anti-inflammatory actions, may explain pravastatin's anti-diabetogenic effect remains elusive. Other mechanisms are also conceivable, for instance direct effects on the endocrine pancreas. *In vitro* studies have shown that lipophilic statins (simvastatin) inhibit glucose-stimulated insulin secretion by blocking voltage-gated L-type Ca^{2+} channels in insulin-secreting β-cells, whereas pravastatin has no such adverse influence (83). Moreover, pravastatin has been reported

to prevent inflammation and rejection of transplanted islets of Langerhans. (84). Interestingly, the administration of pravastatin to female Wistar rats (paper II) resulted in a substantial increase in PBF and IBF. In concordance with this, insulin secretion was also augmented (preferentially late-phase secretion) and glycemia was improved following an IPGTT in pravastatin-treated animals. These findings reveal significant qualitative and quantitative gender-specific differences between male and female rats in terms of vascular and metabolic responses to pravastatin. Along with the beneficial effects above, estrogen may also be implicated in the regulation of pancreatic microcirculation. The existence of gender differences in terms of susceptibility to, and mortality from, a variety of vascular diseases has received increasing attention (85-87). Estrogen is known to increase NO production primarily through up-regulating endothelial NO synthase (eNOS) gene expression (88). There also exists a gender difference in basal NO release by rabbit aorta (89, 90). Alternatively, estrogen may function by up-regulating eNOS and/or enhancing production of eNOS-derived NO, thereby producing a vasodilatory effect (87).

Type 2 diabetic patients frequently have elevated serum levels of free fatty acids, secondary to insulin resistance and increased lipolysis (74, 80, 82, 91). It is commonly believed that free fatty acids are responsible for much of the endothelial dysfunction and vascular damage in type 2 diabetes (74, 80, 82, 91). They may also contribute to β-cell dysfunction and demise through steatosis and apoptosis (collectively termed lipotoxicity (92-95)). Injection of palmitate to male Wistar rats (paper I) induced a preferential and significant suppression of IBF albeit without any effect on non-stimulated serum insulin concentrations or blood glucose. Our results add another means by which free fatty acids may negatively impact β-cell function in diabetes by impeding nutritive islet blood flow and thereby further aggravating the diabetic state by

limiting the supply of insulin needed to curb hyperglycemia. Alternatively, the preferential impairment of islet blood flow induced by palmitate could represent a protective mechanism by which islet exposure to free fatty acid toxicity can be limited.

4.2 PANCREATIC ISLET BLOOD FLOW IS ENHANCED BY ETHANOL (III)

The interaction between alcohol consumption and the pancreas is complex, with both beneficial and adverse effects. Alcohol abuse is widely recognized as an etiological factor in pancreatitis, both acute and chronic. Alcoholism is coupled to elevated incidence and mortality of diabetes and vascular disease (96), and it is also known that alcoholic pancreatitis can lead to impairment of β-cell function resulting in inadequate insulin secretion and glucose intolerance (97). Additionally, extensive clinical experience indicates that unexpected, frequent, and prolonged hypoglycemia is a substantial problem after alcohol ingestion in both type 1 and type 2 diabetic subjects, especially in patients on oral hypoglycemic agents or in malnourished subjects. However, the mechanisms underlying the hypoglycemic properties of ethanol are unknown. Since insulin secretion *in vivo* can be rapidly tuned by changes in pancreatic microcirculation, and ethanol is known to cause vasodilatation, we evaluated the influence of acute alcohol administration on IBF and dynamic changes in insulin secretion and glycemia in male Wistar rats (paper III). Our results indicate that low concentrations of ethanol elicit a substantial stimulation of IBF, augmenting late phase insulin secretion, and induce late hypoglycemia. Because ethanol can increase plasma NO concentrations (98, 99) and NO can dilate blood vessels, the possibility arose that this action of ethanol may be mediated, at least in part, by NO. IBF is extremely sensitive to NO, so a local increase in NO production would be expected to preferentially increase IBF than PBF (51, 84). Indeed, the NO synthesis inhibitor L-NAME blocked the ethanol effects in this study, thus corroborating this assumption.

However, the effects noted in our present work do not seem to reflect a generalized response to the alcohol in the splanchnic bed, because there was no discernable change in perfusion of other abdominal organs, such as the exocrine pancreas or adrenals. Previous work also showed unaffected PBF by either acute or chronic ethanol administration in rats (100), lending support to our present findings. Our results suggest massive redistribution of blood flow within the pancreatic gland in response to alcohol, selectively diverting it to the endocrine part. Ethanol consumption is known to result in a relatively specific reduction in the neural regulation of heart rate by vagal pathways (101). It has been shown that the glucose-induced increase in islet blood flow is medicated by vagal cholinergic influences (48, 102). In our experiments, atropine administration fully blocked the effects of ethanol so it indeed appears the actions of ethanol are medicated by vagal pathways.

In our present observations, none of the treatments influenced non-stimulated serum insulin levels significantly. Blood glucose concentrations also did not differ between any of the treatment groups in the non-stimulated state. Insulin secretion was substantially augmented during an IPGTT late phase and glucose tolerance was significantly improved in the animals treated with ethanol. At the end of the IPGTT (120 min after the 10 sec bolus of ethanol), hypoglycemia was induced in ethanol-treated rats with blood glucose levels averaging 2 mmol/l. Total amount of insulin secreted during the 120 min IPGTT (area under the curve) showed that insulin secretion in rats treated with ethanol was significantly higher than in control rats receiving solvent only; also there was a decrease in post-load glycemia, expressed as AUC. Ethanol has been reported to inhibit insulin secretion *in vitro* in the rat (103), but it acutely augments glucose-stimulated insulin secretion in man (104) perhaps by priming the islets to glycemic stimulation (105). Alternatively, chronic ethanol intake may increase the propensity for insulin resistance and impaired glucose disposal,

characterized by elevated fasting glycemia (106, 107) and postprandial hyperglycemia (108). However, several epidemiologic studies show decreased incidence of new-onset diabetes by alcohol (109, 110) and alcohol has long been known for its hypoglycemic effects when given acutely (111, 112). Late phase hypoglycemia was observed in our present study and we speculate that this was in part caused by the sustained insulin secretion evoked by the heightened islet blood flow elicited by the alcohol.

4.3 GENDER-SPECIFIC REGULATION OF PANCREATIC ISLET BLOOD FLOW BY RAS, PRAVASTATIN, AND FREE FATTY ACIDS IN MALE AND FEMALE DIABETIC GK RATS (IV)

The non-obese GK rat model was developed in 1972 in Sendai, Japan (113)**.** All offspring were frankly diabetic by the 10^{th} generation (114, 115). Glucose-induced insulin secretion is markedly and selectively impaired in the isolated perfused pancreas and isolated islets of GK rats (116-120). Time-dependent histological changes in a minority of GK islets, describes as "star-fish shaped" appearance, were reported to occur with increasing prevalence after the age of 2 months and onwards (113, 121-123). Insulin resistance is moderate in GK rats and appears to develop secondary to hyperglycemia (124). Also, and in sharp contrast to non-diabetic animals, insulin failed to suppress the exaggerated basal hepatic glucose production (HGP) in GK rats, indicating the presence of hepatic insulin resistance (115, 125). GK rats display a markedly increased basal islet blood flow co-existing with basal hyperinsulinemia (126), and on conceptual grounds it may be conceivable that chronic islet hyperperfusion with an attendant rise in capillary blood pressure may lead to structural and functional damage to the islet blood vessels and perhaps also the islets *per se* (35).

The results of paper IV reveal that irbesartan and captopril induced a robust increase in GK rat IBF in both male and female groups. The results confirm our previous findings

with these drugs in normal Wistar rats of both genders (127, 128). Captopril also significantly increased serum insulin concentrations in male GK rats. It is known that locally produced Ang II may suppress IBF (61, 74). An elegant piece of work performed on single perfused islets very recently showed that administration of Ang II and L-NAME contracted islet arterioles, whereas NO and adenosine dilated them (129). Additionally, irbesartan has the capacity to enhance vasodilatation (61, 74). These mechanisms, among others, may have contributed to our current findings.

In this paper, we have also employed another Ang II receptor antagonist, candesartan, to further address the role of pancreatic RAS in the regulation of islet microcirculation. Recent research described that candesartan efficaciously reduced fibrosis in and around the islet and prevented the loss of endothelial cells in diabetic mouse islets by long term treatment. This may indicate that the beneficial influence of candesartan on glucose tolerance noted in clinical trials (82) may in part occur through protection by the drug against progressive β-cell damage and demise (130, 131). Our results show that candesartan increased IBF and PBF significantly only in female GK rats, but not in male animals. This gender difference was not observed with irbesartan and captopril. The underlying mechanism behind this notion remains elusive. However, gender-related differences in the responsiveness to RAS-interfering drugs have been long noticed; in animal research mainly at the tissue level (132-139), while clinical studies have predominantly evaluated gender effects on the circulating, *i.e.* systemic, RAS (140-147). The characterization of an estrogen-responsive element in the 5'-flanking region of the angiotensinogen gene was an important early finding to prove an interaction with sex hormones and the RAS at the molecular level (136, 148). More contemporary research has shown that renin is suppressed by estrogen (146). Collectively, these studies are in line with the observation that plasma renin levels are lower in women compared with men (146, 149). AT1 receptors may also be down-

regulated by estrogen since estrogen deficiency leads to up-regulation of these receptors (138). In female transgenic rats with activated RAS, estrogen treatment confers a beneficial effect against hypertension by amplifying the vasodilator contributions of Ang-(1-7) and reducing the formation and vasoconstrictor actions of Ang II (150). Obviously, any of these mechanisms (or others) could contribute to the gender-related differences noted in our *in vivo* islet model.

Pravastatin induced a substantial increase in IBF and PBF, as well as a significant rise in serum insulin levels in male GK rats, as expected. In contrast, in female GK rats PBF was augmented by the drug but without any corresponding impact on IBF. In our previews finding in female Wistar rats, pravastatin actually increased both IBF and total PBF in the same time (127). The reason for this difference is currently unknown. However, in diabetic animals the vascular response to glucose is known to be amplified in islet arterioles (129), indicating enhanced islet blood perfusion in diabetes. Also, with increasing age, persistent hyperglycemia may cause islet hypoperfusion in GK rats (151). The islet blood hyperperfusion is accompanied by an islet capillary hypertension. Such shear stress changes are known to change the gene expression of surrounding cells (151), then probably further impacting islet function. Such mechanisms could possibly contribute to our results. Additionally, dyslipidemia induces impaired endothelium-mediated vasodilatation (76, 152). Pravastatin has beneficial anti-inflammatory effects on endothelial function (75, 77, 78, 153), and therefore may significantly influence selective tissue perfusion.

Palmitate decreased both PBF and IBF in GK rats of both genders. These results confirm our previous study in non-diabetic rats (128). This might be one explanation by which free fatty acids may adversely affect β-cell function, at least by part, through reducing IBF and thereby limiting the supply of insulin needed in peripheral organs.

When making comparisons with the diabetes preventive actions of pravastatin and RAS-interfering drugs noted in clinical trials, it should be kept in mind that our present results reflect only very acute effects of the substances tested. While reports on long-term effects of such drugs on islet blood flow are scarce, this interesting issue is worthy of further investigation.

5 CONCLUSIONS

- A local RAS exists in the rat pancreas and regulates both exocrine and endocrine microcirculation in this gland. Vasoactive drugs, which are frequently given to diabetic patients, may rapidly and preferentially stimulate pancreatic islet blood flow, augment insulin secretion, and, therefore, improve glucose tolerance.
- Substantial gender differences exist in the vascular and metabolic responses to these drugs in both normal and diabetic animals, which might be related to the ability of estrogen to influence vasodilation.
- Free fatty acids, which are frequently elevated in type 2 diabetic patients, may contribute to an impaired nutritive islet blood flow and thereby further aggravate the diabetic state by limiting the supply of insulin needed to curb hyperglycemia. Alternatively, the decreased islet blood flow could represent a protective mechanism by which islet exposure to free fatty acid toxicity can be limited.
- Ethanol acutely exerts substantial influences on pancreatic microcirculation by evoking a massive redistribution of pancreatic blood flow from the exocrine into the endocrine part via mechanisms mediated by nitric oxide and vagal stimuli, augmenting late phase insulin secretion, and thereby evoking hypoglycemia. This effect may in part underlie the well known hypoglycemic properties of alcohol in diabetic patients or in alcoholics with hepatic failure.
- Collectively, these direct islet effects of RAS-interfering drugs, statins, fatty acids and ethanol may prove valuable in designing different and gender-specific treatment strategies for diabetic patients or subjects at risk of developing glucose intolerance. Moreover, vasoactive drugs that are frequently given to diabetic patients may confer additional treatment benefits beyond their systemic effects by increasing islet blood flow, augmenting insulin secretion and improving glycemia.

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