UPSALA JOURNAL OF MEDICAL SCIENCES, 2016
VOL. 121, NO. 2, 81–95
http://dx.doi.org/10.3109/03009734.2016.1164769

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REVIEW ARTICLE

OPEN ACCESS

Pancreatic islet blood flow and its measurement

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ABSTRACT

Pancreatic islets are richly vascularized, and islet blood vessels are uniquely adapted to maintain and support the internal milieu of the islets favoring normal endocrine function. Islet blood flow is normally very high compared with that to the exocrine pancreas and is autonomously regulated through complex interactions between the nervous system, metabolites from insulin secreting β-cells, endothelium-derived mediators, and hormones. The islet blood flow is normally coupled to the needs for insulin release and is usually disturbed during glucose intolerance and overt diabetes. The present review provides a brief background on islet vascular function and especially focuses on available techniques to measure islet blood perfusion. The gold standard for islet blood flow measurements in experimental animals is the microsphere technique, and its advantages and disadvantages will be discussed. In humans there are still no methods to measure islet blood flow selectively, but new developments in radiological techniques hold great hopes for the future.

ARTICLE HISTORY
Received 3 March 2016
Accepted 8 March 2016

KEYWORDS
Blood flow measurements; islet blood flow; microspheres; pancreatic islets

In 1960 Claes Hellerström and co-workers published a paper in which they tried to assess the β-cell activity by quantifying the number of erythrocytes in the islets (1). A correlation between islet endocrine activity and blood flow was assumed, but this was difficult to demonstrate due to lack of precision in the method. He did not, however, forget the topic, and 20 years later in 1980 one of the authors (L.J.) of the present review began studying the islet vasculature under the benevolent and extremely stimulating guidance of Claes.

Claes, you were, as always, correct! There is a correlation between islet endocrine activity and blood flow, and you helped to develop this field of research. We aim to give a brief overview of the islet vasculature, and focus on the methods that can be used to measure its blood flow, discussing the advantages and disadvantages with the various techniques.

1. Pancreatic islet morphology

A. Cellular composition

In't Veld and Marichal have provided an excellent overview of pancreatic islet morphology (2) where further references can be found. In most vertebrates pancreatic islets are distributed throughout the pancreas in cellular aggregates containing from a few up to several thousands of cells. They are demarcated by a connective tissue capsule, the extent of which is species-dependent. Most focus on islet function has been directed towards their endocrine cells, in particular the insulin-producing β-cells. These cells constitute 40%–70% of all endocrine cells in the islets, depending on species. Common laboratory animals, i.e. mice and rats, have more β-cells (60%–70%) than humans (40%–50%) (3). The other islet endocrine cells are glucagon-producing α-cells, somatostatin-producing δ-cells, pancreatic polypeptide-producing PP-cells, and ghrelin-producing ε-cells. Most islets contain a mixture of these cells. A notable exception is the uneven distribution of PP- and α-cells, where the former are found mainly in the caput region, i.e. the former embryonic ventral anlage, whereas α-cells are found mainly in the tail region, corresponding to the dorsal anlage. β-Cells are more evenly distributed in the pancreas, even though humans seem to possess occasional β-cell-rare islets. It should be noted that smaller islets contain predominantly β-cells, whilst the other endocrine cell types are mainly found in the larger islets.

The reasons for the coexistence of these hormone-producing cells in discrete micro-organs are not known for certain (4), and we will briefly return to this issue later, since we believe that this organization profoundly affects the interactions between endocrine cells and islet vasculature to achieve a fine-tuning of islet hormone release.

Besides the endocrine cells, the islets contain numerous other cell types, vascular cells, other stromal cells, immune cells, and neural elements (Table 1). The present review focuses mainly on the blood perfusion of the islets, and we will therefore discuss in more detail the cells associated with the islet vasculature. Nervous elements in the islets consist of nerve cells with axons, referred to as neuro-insular complexes type 1 when there are nerve cells bodies, and type II when

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Table 1. Cell types in pancreatic islets.

Cell types	
Endocrine cells	α-cells β-cells δ-cells ε-cells PP-cells
Stromal cells	Fibroblasts Myofibroblasts Stellate cells Cajal cells Occasional duct cells
Vascular cells	Endothelial cells Vascular smooth muscle cells Pericytes Adventitial stromal cells
Immune cells	Granulocytes Lymphocytes Macrophages Dendritic cells Mast cells
Neural cells	Neurons Schwann cells

Data from (2,20).

only intra-islet axons are found (5,6). Many of these nerves affect not only hormones secretion per se, but also islet blood flow. The Schwann cells should be mentioned in this context, since they seem to surround all islets in an envelope, but their functions remain enigmatic (7). It has been suggested that they may participate in regenerative processes after islet transplantation (8).

Immune cells can be numerous during islet inflammations, i.e. insulitis, but are normally sparse in the islets. Interestingly, it has recently been suggested that recruitment of different subtypes of macrophages may influence the vascularization of islets, especially after transplantation (9,10). However, these notions are beyond the scope of this review.

Among stromal cells fibroblasts contribute by forming the thin network of reticular and collagenous fibers in the islets as well as the capsule surrounding each islet. Normally also pancreatic stellate cells with their characteristic lipid droplets can be seen (11). Their exact function in the islets is unknown, but their numbers are increased during desmoplastic reactions to pancreatic adenocarcinomas as well as during chronic pancreatitis (12). In both these instances they contribute to the pancreatic fibrosis characteristic of these conditions. We have also noted that their numbers in islets are increased in GK rats, a type 2 diabetes model characterized by islet fibrosis (11). There are myofibroblasts present under these conditions as well (13), but whether they emanate from the stellate cells or other cell types is unknown. Another stromal cell in islets is the interstitial cell of Cajal, a cell of neural origin. Such cells are common in the intestine where they serve as pacemaker cells, being active in regulation of intestinal motility (13). Whether such effects occur also in the pancreas is an intriguing possibility, but so far unknown (14).

B. Morphology of pancreatic islet vasculature

This subject has been highlighted in several reviews (2,15–21). The islets contain fenestrated capillaries, which

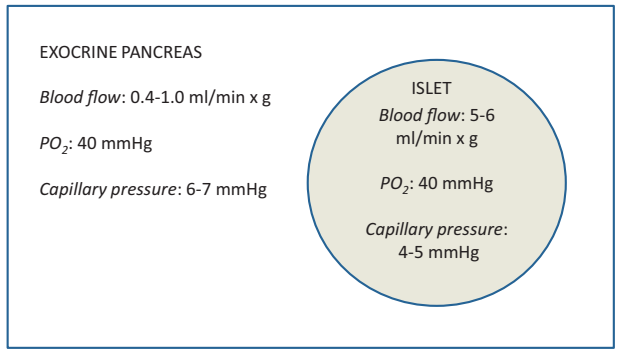

Figure 1. Blood flow values in exocrine and endocrine pancreatic parenchyma. Modified from Jansson and Carlsson (15).

constitute 8%–10% of the islet volume, and are organized into a glomerular-like network, described already in the 1880s (22), i.e. at a time when islet function was unknown. The number of fenestrae is approximately 10 times higher than in exocrine pancreatic capillaries (23) and is induced by the high local production of vascular endothelial growth factor-A (VEGF-A) from the islet β-cells (24,25). Thus, after transplantation when islets are implanted into organs with continuous capillaries, the ingrowing new microvessels initially form such capillaries, but already after a few days the endothelium becomes fenestrated (26). It is likely that the fenestrations facilitate hormonal passage into the circulation as well as participate in draining extracellular fluid analogous to functions normally performed by lymphatic capillaries (27). Besides being fenestrated, islet capillaries are wider than those in the exocrine pancreas, having a 20%–30% larger diameter, and their vascular density is much higher (23). It can be noted that after transplantation the vascular density of the islet grafts in sites such as the liver is less than that in endogenous islets for several months post-transplantation (15,28).

Pericytes located between the endothelium and the capillary basement membrane can occasionally be found, but they are not particularly numerous (8,29). Their functions in islets are little studied and largely unknown. It has been hypothesized that they contribute to angiogenesis or as contractile cells (8), but these notions are mainly conjectural.

The larger islets contain one to three afferent arterioles, which originally branch from intralobular arteries in the exocrine tissue. These afferent arterioles sometimes contribute tributaries also to exocrine acini, but many of them supply only the islets (16). Smaller islets are directly incorporated into the capillary network of the exocrine pancreas. The organization of larger islets means that there is an anatomical correlate, which enables autonomic regulation of the islet blood perfusion. The diameter of the afferent arteriole is usually 20–30 μm (Figure 1), and it contains one, or occasionally two, layers of vascular smooth muscle cells (VSMC), which are surrounded by adventitial tissue containing fibroblasts, but also occasional adipocytes (30).

The anatomy of the veins draining the islets is to some extent species-dependent and also depends on the size of the islets. As mentioned above, smaller islets drain with exocrine veins, whereas larger islets have a different organization (16). Most common is a direct venous drainage to one to four larger veins with a basket-like network of smaller vessels lying

in association with the connective tissue capsule at the interface between the islets and the surrounding exocrine tissue. These veins then empty into intralobular veins and, finally, into the portal vein. However, some of the islets, particularly in larger mammals, have a so-called insulo-acinar portal system. This means that the islet capillaries empty into numerous small venulae, which then anastomose with capillaries in the exocrine pancreas. By such means there are two serially connected capillary systems, i.e. a portal system. In rodents this is of minor importance, but its role in human islets, as well as other primates, is likely to be larger even though this is little studied (16,31).

In general, islets are devoid of lymphatic capillaries, whereas the exocrine pancreas contains numerous such vessels (27,32). Lymphatics draining the pancreas contain insulin, but at a concentration <30% of that seen in the portal vein (33). To what extent this represents recirculation of insulin is unknown. In islet grafts, lymphatics initially develop, but with time they decline in number (34). These findings have been interpreted to reflect that the highly permeable islet blood capillaries, as well as the small size of the islets, preclude the need for lymphatic drainage. Other endocrine organs, such as the pituitary and parathyroid glands contain lymphatics, but they are much larger than those of islets, and the need to prevent prolonged release of hormones into the circulation by the lymphatics is not present. From an evolutionary point of view, the Brockman bodies seen in fish, i.e. centimeter-large accumulations of islet tissue, contain lymphatics (35).

2. Functional implications of islet morphology on blood flow regulation

All functional evidence suggests that islet blood flow is regulated autonomously from that of the exocrine pancreas and that this regulation occurs at a pre-capillary level, i.e. in the arteriole (36); for a summary see Brunicardi et al. (21,37). There is no evidence that post-capillary regulation located to the draining veins occurs (38). In this section we will provide a more detailed overview of the sites that we believe are of importance for islet blood flow regulation.

A. Arterioles

The presence of VSMC in the arterioles provides the basis for the separate regulation of islet blood flow. Both arterioles and the endocrine cells themselves receive efferent nerves, particularly sympathetic nerve fibers, but also non-adrenergic, non-cholinergic (NANC) nerves (5,6,39). The latter are presumably purinergic and/or nitric oxide (NO)-containing nerves (40). Furthermore, afferent sensory nerves affecting blood perfusion (41) can also be found within the islets (42). A summary of the effects of nerves on islet blood flow and insulin secretion is provided in Table 2.

Arteriolar VSMC are also responsive to most locally produced endothelium-derived vasoactive substances but can be affected by substances, including hormones, reaching them through the systemic circulation as well. Islet endothelium, mainly in arterioles but also capillaries, produces both NO and endothelin-1 (30,43) and probably several other mediators that may directly influence the VSMC. The prime importance of NO for islet blood regulation has been repeatedly demonstrated (44,45).

Table 2. Effects of different neurotransmitter substances on insulin release and islet blood flow (IBF).

	Insulin	IBF
Muscarinic (M3) receptors	+	+[a]
β_1 Adrenoceptors	0	0
β_2 Adrenoceptors	+	–
β_3 Adrenoceptors	0	+[b]
α_1 Adrenoceptors	–	+
α_2 Adrenoceptors	0	0[b]
D2 receptors	–	+[b]
D3 receptors	0	0

Data from (5,95,166,167).
[a]Inhibitory effect during hyperlipidemia.
[b]Normalizes increased basal islet blood flow in GK rats, a type 2 diabetes model.

During the last few years, increased focus has been directed against the adventitia of resistance vessels, since e.g. adipocytes, macrophages, and other cells found here may also produce substances, such as adipokines, cytokines, and gaseous transmitters, which may affect VSMC through intravessel paracrine mechanisms. We have for instance observed that GK rats, a type 2 diabetes (T2D) model, express more interleukin-6 in their adventitia than normal rats (unpublished observation). A further influence on arteriolar VSMC may be derived from substances produced by adjacent endocrine cells. It should be noted that some arterioles branch at the periphery of the islets, whilst others penetrate into the islet core before forming into capillaries (16,37,46). This means that a variable part of the arteriole is exposed to high concentrations of substances produced within the islets. These substances include islet hormones and factors co-released with endocrine granules, e.g. chromogranins, synaptophysins, and Zn^{2+} (47). Furthermore, other substances produced by metabolically active endocrine cells, namely nucleotides such as ATP, ADP, and adenosine, may also be present in high concentrations close to the VSMC (48). It is easy to envisage that such substances may affect both endothelial cells and VSMC in the arterioles and thereby affect blood flow. However, to what extent they may also affect ionic channels in capillary endothelial cells and then transmit retrograde signals to the arterioles (49) is an intriguing concept, but one not yet fully explored.

B. Capillaries

Since capillaries consist of a tube of single endothelial cells surrounded by a basement membrane, they do not in general possess any blood flow regulatory properties. No signs of any pre-capillary sphincters have been noted in either islet arterioles or proximal capillaries, so it seems likely that the flow regulation is indeed mainly restricted to the arterioles. There are occasional pericytes (29,50), but their contractility and participation in flow regulation in mammals are controversial.

Morphological studies of islet capillaries, as well as studies on isolated islet endothelial cells, have not demonstrated any

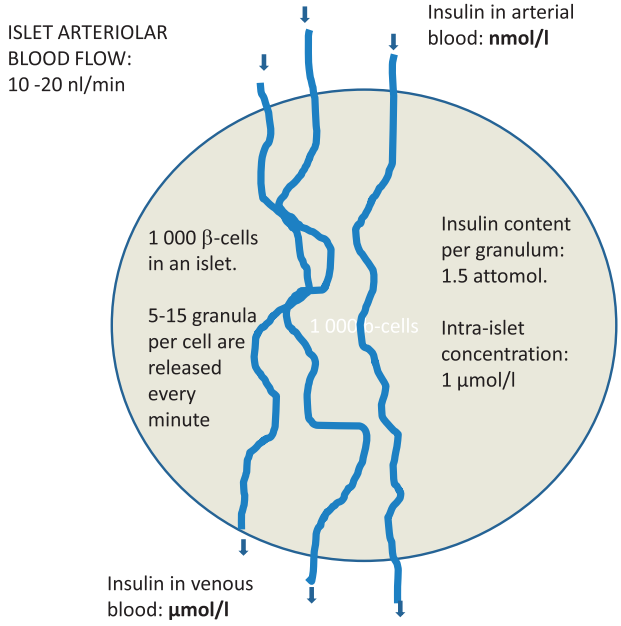

Figure 2. Estimation of intra-islet insulin concentrations based on previously published data on insulin release (168) and islet blood flow (87).

heterogeneity between individual islets. The organization of the islet capillaries is usually described as glomerulus-like, and islets are highly vascular, although not as profound in human as in rodent islets (51,52). Most β-cells have at least parts of their cytoplasm facing a capillary, and the endocrine granules are preferentially located in this part of the cytoplasm (53). This seems to be the case also for the other islet endocrine cells, with the possible exception of δ-cells. The latter cell type contains long, slender processes which establish contact with other cell types and to some extent also to the islet capillaries (54).

This endocrine cell polarity provides the basis for the B-A-D flow hypothesis formed on morphological observations and perfusion studies in rodent islets, but has also been suggested to be valid for human islets (55). Rodent islets have a β-cell-rich core surrounded by a mantle of non-β-cells. The arterioles are described as entering the islets through a discontinuity in this mantle, and then branch into capillaries in the β-cell-rich core of the islets (Figure 2). This would mean that high local insulin concentrations are transported in the blood towards the periphery of the islets, where α-cells would add glucagon and, finally, the δ-cells their somatostatin. Paracrine/endocrine interactions between the islet endocrine cells are thereby, at least partially, mediated by the flow direction in the islets (56). Besides these morphological studies, also functional studies with retrograde perfusions corroborating these notions have been performed (55). It is worthy of note, however, that this theory has been disputed (21). Indeed, there have also been suggestions that the blood flow direction instead is from the periphery towards the center or from 'top to bottom' without any direct correlate to micro-regions of different endocrine cells (21,37). In an elegant study Nyman and co-workers (46) examined this in detail in mice and found that all of these patterns could be found, but that core-to-mantle constituted approximately 50% of the studied islets. For technical reasons these studies were confined to larger islets. We have in a series of experiments in rats retrogradely injected microspheres and confirmed the findings of Nyman and co-workers (unpublished observations).

C. Veins

The morphology of the venous drainage has been mentioned in the preceding section. The morphology of the veins themselves is similar to that of other veins and venulae in the body, and as expected no valves have been described. Venous VSMC are sparse, and the vessel size is quite variable. Venulae associated with insulo-acinar portal systems are small and have a diameter of only 15–20 µm (57). There is no evidence of any flow control by the activity of venular VSMC (38). An interesting observation is that when islets are transplanted the graft is drained exclusively by larger veins, and no signs of an insulo-acinar portal system can be discerned (58). Another issue of importance is that islet inflammation, such as insulitis, which is associated with a mononuclear cellular infiltration, seems to be initiated in the islet periphery (59,60), i.e. at the location of the veins. In the diabetes-prone BioBreeding/Worcester rat, an animal model of type 1 diabetes, an islet venular defect has also been described (61). Moreover, occasional mast cells, which affect venular permeability, can be found at this location (62), and the number of mast cells is increased in T2D (63). After islet transplantation, so-called 'blood lakes' are formed, which probably emanate from disrupted veins. They restitute and become replaced by connective tissue a few days post-transplantation (15).

D. Islet cellular composition affecting vascular morphology

The islet vascular architecture referred to above is probably not static, but is likely to vary not only between different islets but also over time and depending upon the functional demands on the endocrine cells (64). It has been known that smaller islets in rodents with diameters <100 µm mainly contain β-cells (65). These islets seem to be incorporated into the exocrine capillary system (66). This implies that the regulation of islet blood flow in smaller islets is probably not autonomic from that of the exocrine pancreas, but is regulated in concert with the latter. It should be noted, however, that the contribution of the smaller islets to the total pancreatic islet volume is small (65), so a majority of islet endocrine cells are located to islets with an autonomous blood flow regulation. Human islets sometimes consist of many α-cells, but the blood flow regulation of such islets is at present unknown. An interesting comparative physiological approach in this context is that some birds, e.g. ducks, have islets containing predominantly α-cells, and the vasculature of such islets differs from that of β-cell-containing islets (67).

Another factor which affects not only the cellular composition per se but also islet blood flow is age. Before birth islets are small and very few islet capillaries have developed, but in the post-partum period islet endocrine and vascular mass both rapidly expand (68). Nevertheless, the blood perfusion

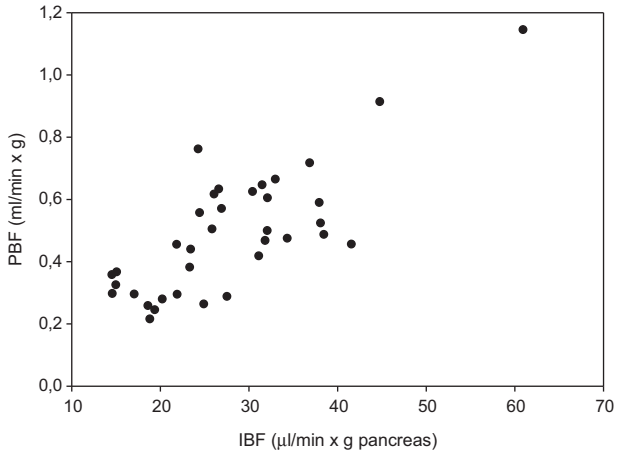

Figure 3. Correlation between islet blood flow (IBF) and total pancreatic blood flow (PBF) on anesthetized normoglycemic Sprague-Dawley rats. Data are based on 36 recent control experiments. (Pancreatic blood flow $=0.0466+(0.0158\times$ islet blood flow)). $P<0.001$.

in 5-week-old animals is considerably lower than in older animals. For instance, the fractional islet blood flow in 5-week-old rats is only 1%–2%, which increases to 8%–10% after 15 weeks, 15% after 12 months, and >20% at 2 years of age (69–71). Similar findings have been made in mice (72). This increase is present also when adjusted for changes in islet volume. We believe that this reflects increased insulin resistance with age, but the exact mechanisms behind the increase are not known, even though it seems likely that NO is involved (73).

When islet growth is stimulated as after partial pancreatectomy (74,75) and during pregnancy (76) there is usually an increased islet blood perfusion. A controversial issue in the context of islet growth is to what extent new islets may form by 'budding' from precursor cells in smaller pancreatic ducts, an issue previously taken for granted (77,78), but lately refuted (79). There are some older studies suggesting that peri-ductular islets derive their arterial blood from ductal blood vessels (80), and it has even been suggested that ductal blood vessels constitute a third capillary network in an intra-pancreatic portal system (81). In that latter study the authors described that the first system would be in islets, the second in exocrine tissue, and, as mentioned, a third in ducts. Such islets would probably contribute very little to total islet volume, and therefore be of little importance.

E. Pancreatic blood flow and heterogeneity between islets

When referring to blood flow values in the exocrine and endocrine pancreas we use values obtained by the microsphere technique. The blood perfusion of the pancreatic islets is considerably higher than that of the exocrine gland, and it is, in adult rats, 5%–20% of total pancreatic blood, despite the islet volume constituting only 1%–2% of the pancreas. Normally, there is a close correlation between islet blood flow and total pancreatic blood flow (Figure 3), but during e.g. hyperglycemia their responses become dissociated (82,83). When correcting for weight, the corresponding value for islet blood flow is 5–6 mL/min × g islets (Figure 1), which is one of the higher blood flow values in the body, normally only surpassed by that to the oxygen-sensing glomus cells in the carotid body (84). Very high flow values were recorded also in other species such as for instance Atlantic hagfish (85), mouse (86), and rabbit (87). There are, however, no differences in blood perfusion between islets in the head region of the gland, i.e. pancreatic polypeptide-rich islets supplied by the superior mesenteric artery, and those in the tail region which are glucagon-rich and receive their blood from the celiac trunk (88). This holds true for the exocrine pancreas as well, which despite its dual arterial supply regulates its blood flow synchronously (89).

Of interest in this context is whether this high blood perfusion involves all islets, or only some of them, which would then constitute an extremely well-perfused and presumably functionally more active subgroup. Indeed, repeated microsphere injections have indicated that microspheres predominantly end up in the same 10% of islets (90), and that these islets further increase their blood perfusion in response to glucose (90). Injection of a marker of low oxygenation, pimonidazole, demonstrated that 20%–25% of the islets are normally poorly oxygenated and with less protein biosynthesis than others, but become better oxygenized at increasing demands for insulin release (28). With a keen eye to its limitations the microsphere technique has been used to identify tentatively the most highly perfused islets, and differences in vascular density, glucose metabolism, β-cell proliferation, and insulin release have been identified, suggesting that there is indeed a coupling between islet blood flow and metabolic activity (91,92). It is worthy of note, however, that only insulin has been studied in this context, and it is unknown if such relationships exist also for other islet hormones. The observation of a highly perfused subgroup of islets opens the intriguing notion that islets receiving lower blood flow may constitute a functional reserve, which can increase their flow and thereby their functional activity upon demand. It is tempting to speculate that this may be a safety measure to prevent an excessive insulin release at any given time. It would also provide a twist to the question raised for many years (4), why β-cells are distributed into a vast number of tiny cell aggregates rather than forming a large, compact gland like most other endocrine organs.

3. Techniques used to study islet blood flow

The high islet blood flow is normally further increased whenever there is a need for increased insulin secretion, and this is assured by complex interactions between several regulatory mechanisms. Furthermore, conditions with decreased glucose tolerance, when there is a need for augmented insulin release, are also associated with an increased islet blood flow (89), and such factors ensuring these blood flow responses include:

1. Metabolic factors from β-cells: We have noted that especially adenosine and ADP seems to be of major importance for this, and directly couple the degree of

metabolism to the required blood flow increase (93,94).

2. Nervous system: The vagus nerve, sympathetic nerves, and sensory nerves all affect islet blood flow, and their effects are summarized in Table 2 (95).
3. Endothelium-derived constricting and dilating factors: So far approximately 10 different substances in this category have been examined, and the responses are as expected. The pronounced sensitivity for NO shows its major importance for islet blood flow regulation (44).
4. Hormones: Islet hormones, incretins, and adipokines are potential regulators of islet blood flow. With the exception of incretin hormones, there are only sparse effects of hormones on islet blood flow, but incretins seem to potentiate glucose-induced stimulation of islet blood flow (96).

These blood flow regulatory mechanisms are very similar to those mediating responses also in other tissues, and especially so to those mediating postprandial reactive hyperemia in the gut (97). However, with regard to the islets it seems as if the major factor affecting islet blood flow is the prevalent blood glucose concentration, and this may be by activating one or several of the factors referred to above (83,90,98). This would then modulate the blood perfusion according to the needs of the insulin-secreting β-cells. To the best of our knowledge there is no similar coupling between the release of the other islet hormones and blood flow.

The distribution of the islets within the exocrine parenchyma means that measurements of total pancreatic blood flow is not sufficient, since the fraction of the blood flow being diverted to the islets is unknown. Therefore, measurements must separately register endocrine and exocrine blood flow. This precludes the use of several common methods used to assess regional blood circulation, such as those based on the Fick principle, or electro-magnetic or ultrasound flow probes placed around arteries or veins in the organ. These techniques can only determine total pancreatic blood flow, and since the pancreas has multiple arterial supplies, both the superior mesenteric artery and the celiac trunk, this necessitates the placement of at least two probes (89). Also techniques based on radiological methods such as magnetic resonance imaging (MRI) and positron emission tomography (PET), which have the added benefit of being applicable to humans, provide measurements only of total pancreatic blood flow, mainly due to the fact that the resolution is not sufficiently high to allow visualization of individual islets. However, these problems are at present being overcome, and in the foreseeable future radiological techniques may allow visualization of the islet organ to allow not only determinations of islet blood perfusion, but even more importantly to quantify islet mass in patients (99). Several techniques have been experimentally applied to assess islet blood flow, almost exclusively in rodents and mainly in rats. The predominant techniques that have been used to investigate the blood perfusion of endogenous islets are different *in vivo* microscopy applications, microsphere measurements, and most recently hydrogen gas clearance. Studies on transplanted islets have applied mainly *in vivo* microscopy and laser-Doppler flowmetry, but also microspheres have been tried (15,100). We will now comment on advantages and disadvantages with these techniques with a major focus on measurements in endogenous islets.

A. Microsphere measurements of islet blood flow

This technique is a variant of the deposition techniques (see below under D). Then, instead of chemical substances, small polystyrene particles (commonly referred to as microspheres) are injected into the arterial blood stream. They are distributed into the different capillary beds, where they become entrapped. In this manner, nutritive blood perfusion rather than plasma perfusion is measured, since the microspheres distribute as erythrocytes. The number of microspheres within each organ is proportional to their blood perfusion, and this can be assessed by quantification of their numbers, either directly by counting or by assessing their labels, which can be radioactivity, fluorescence, or different colors (101).

The first experiments applying entrapped particles to measure local blood flow were carried out with starch particles in pigs (102), but other materials such as ceramics have been used. The technique in its present form was introduced in 1967, when isotope-labeled plastic particles with a diameter of 50 μm were injected into fetal lambs (102). This demonstrated that, in comparison with antipyrine measurements, these microspheres did not recirculate to any significant extent, distributed in proportion to the blood flow, and did not affect the circulation physiology in the fetuses, i.e. fulfilled the basic criteria of the microsphere technique. The year after, the concept of an arterial reference sample had been introduced (103), which made measurements of cardiac output with this technique possible. Since then this application has developed into the gold standard for regional and intra-organ blood flow measurements (101,104–106).

The adequate use of microspheres necessitates the fulfillment of several criteria, namely: 1) Adequate mixing of the microspheres with blood in the central circulation; 2) Complete extraction of microspheres during the first passage through the tissues; 3) Flow properties similar to those of red cells; 4) No circulatory artifacts should be induced by the microspheres; 5) The microspheres (or their marker if they are labeled with something) should remain in the tissues; and 6) The measuring accuracy should be sufficient.

Most islet blood flow studies, which were first performed in the early 1980s (71,83,87) have used polystyrene plastic particles with a diameter of 10 μm (or occasionally 15 μm) stained black or other colors. Fluorescence-labeled microspheres have been used as well (91,92), whilst microspheres with radioactive tracers have been rarely used, even though they have been commonly utilized for measurements of other organ blood flow values (107).

(i) Criteria for the adequate use of microspheres. It is important that the microspheres are adequately mixed with the arterial circulation and occupy the entire vessel profile, so that their disposal into the tissues mimics that of red blood cells. This can most easily be achieved by administering the

microspheres where arterial blood flow is turbulent, i.e. into the left atrium or ventricle or in the ascending aorta. Indeed, the heart is the only choice if myocardial blood flow is to be studied, since the coronary arteries branch immediately above the aortic valves. In studies in rats less variation in the flow determinations were seen after intra-atrial injections (108).

However, in small animals such as rats and mice cannulation of especially the left atrium, but also the left ventricle, can be difficult. An alternative experimental maneuver may be to give the microspheres via cardiac puncture, an approach chosen in Mongolian gerbils (109). The placement of the catheter within the heart can be determined by pressure recordings during catheter insertion. There is, however, always a risk of complications by e.g. damage to the aortic valves, since the catheters used for microsphere injections should not be too thin. A diameter of 1 mm is usually required in order to avoid impaction of the microspheres. We usually give the injections into the ascending aorta. We advance the catheter down to the plane of the aortic valves, which can be felt, and then withdraw it 1–2 mm. At this site there is no interference with aortic flow or cardiac output. Furthermore, the precision of blood flow determinations in the pancreas is similar as when applying intraventricular microsphere injections (110). As a rule of thumb intra-aortic microsphere injections provide adequate precision for blood flow determinations in organs below the diaphragm, whereas for organs above the diaphragm administration into the heart is necessary.

Another prerequisite for adequate mixing is that the microspheres do not adhere to one another and form larger aggregates in the microcirculation corresponding to large chains of microspheres. The latter was observed in some initial studies where microsphere behavior in the hamster cheek pouch microcirculation was studied with intravital microscopy (111). Formation of such aggregates or chains is prevented today by addition of Tween to the microsphere solutions.

In all experiments applying microspheres it is desirable to investigate the microsphere content in paired organs, such as the kidneys or adrenal glands. Since we count the microspheres in a microscope (see below) we prefer the latter for logistic reasons, i.e. there is a smaller number of microspheres to count. The number of microspheres found is usually in the order of $>$500 per gland, and this should not differ by more than 10% between the glands if adequate mixing has occurred.

(ii) Complete extraction during first passing. This is of utmost importance, since recirculation of the microspheres will provide erroneous results, with overestimations of blood flow in well-perfused organs. To avoid this, the microspheres should have a size which is larger than the smallest arterioles and capillaries of the organ. Initial studies of microspheres in general led to the conclusion that 15 μm microspheres should be chosen, since their general systemic shunting is small. It has also been shown that the distribution of microspheres of this size correlates well with the distribution of labeled red blood cells (112).

Thus, to minimize shunting of microspheres through capillaries of an organ, a microsphere size as large as possible should preferentially be used. However, this imposes some problems. Firstly, it is advantageous to have as many microspheres as possible within the sample to increase the accuracy of the flow measurement (113). Furthermore, smaller microspheres end up in smaller arterioles/metarterioles and thereby increase the resolution of the flow measurement (83). Many studies on microsphere shunting are from the early days of microsphere usage. These batches were very variable with regard to size; for instance 15 μm microspheres were actually in the range of 15 ± 5 μm (mean ± standard deviation), and even larger size heterogeneity occurred. The result of this was that there was a predominance of larger microspheres in the peripheral tissues, whilst the smaller sizes recirculated. This would of course produce errors especially with regard to intra-organ flow distribution (104). At present microsphere batches are much more homogeneous, and those with a diameter of 10 μm have a standard deviation of approximately 0.2 μm.

A simple way of assessing the total degree of shunting of microspheres in the whole body is to measure the amount of microspheres in the lungs. The normal bronchial circulation is $<$1% of cardiac output, so microspheres exceeding that amount in the lungs represent microspheres shunted in the periphery. With the use of 10 μm microspheres in rats, approximately 2% of the injected microspheres are found at this location (110). In the whole vascularly perfused pancreas approximately 3%–4% of the microspheres enter the portal vein (114). Granted, this is in the whole pancreas, and we do not know for sure whether there are any direct shunts through the islets. There are anecdotic reports that this may occur (100). However, no reports from *in vivo* microscopy of islets have described any shunting of microspheres through or around the islets. In this context we would like to stress that, as outlined in the next section, the microspheres are supposed to mimic red cells, i.e. produce a measure of nutritive blood flow. If there is shunting allowing red cells to bypass the islets, it still means that the microspheres measure the nutritive blood flow.

(iii) Flow properties similar to those of red blood cells. An issue of importance in this context is so-called 'skimming' of microspheres. This was originally described in kidneys, where microspheres traveled centrally in interlobular arteries, and thereby erroneously preferentially accumulated in the outer regions of the cortex (115,116). This is also referred to as steric restriction affecting the distribution of the microspheres. If considering the vascular anatomy in the pancreas, this seems unlikely to occur in this organ, and skimming is not of importance for most organs in the body.

The microsphere size we prefer to use, i.e. 10 μm, is similar to the diameter of red cells. The likelihood of skimming increases with size (115), as well as the simple fact that microspheres become too large to enter some arterioles if they are large enough. Islet arterioles are usually 30 μm (30), and we have indeed noted a decreased islet blood flow if microsphere size is increased (83). The question is in which arteriolar size microspheres of different diameter become entrapped. Initial studies with *in vivo* microscopy of hamster cheek pouches and bat wings demonstrated that the arterioles with impacted microspheres were surprisingly large, namely in the order of 20–60 μm (111,117,118). It was also noted that some 10 μm microspheres could slip through the

capillaries (118). When examining rodent pancreases injected with ink, it seems as if 10 μm microspheres will be found in islet arterioles of a size only marginally larger than the microspheres themselves. When microspheres of different sizes were used to determine arteriolar size, there were some uncertainties in the precision of these estimates (119). For reasons provided below, we believe that designed islet microspheres are representative of islet blood perfusion per se, and do not represent microspheres that have erroneously entered islet capillaries.

(iv) On circulatory artifacts induced by microspheres. Major factors to take into account in this context are the number and size of the injected microspheres. The microspheres that become entrapped in the regional vascular beds act as emboli. This means that, depending on their number and location, they may induce local ischemia and disturb both the microcirculation as well as the general circulation. The number of microspheres that can be injected without causing any undue effects on circulation has been the subject of numerous studies in the past (101,105,113). However, since the size uniformity of microsphere batches has improved markedly in recent years, with absence of erroneously large microspheres, many previously encountered problems have diminished. In early studies of the microsphere technique a total number varying between 60,000 and 320,000 15 μm and 10 μm microspheres, respectively, was reported not to induce any hemodynamic disturbances in rats (120,121). Occasionally, but not always, more than one million 10 μm microspheres could be given without adverse effects (121). We are consistently injecting 300,000 microspheres with a diameter of 10 μm in 300-g rats and 100,000 in mice without ever observing any hemodynamic instability.

It is, however, essential to monitor basal circulatory parameters and blood gases to detect circulatory alterations. We have evaluated the pancreas and islets microscopically for signs of necrosis caused by embolizing microspheres without noticing any such signs. However, if the number of injected microspheres exceeded one million there was some exocrine ischemic damage, whilst the islets remained unaffected (unpublished observations).

It is also relevant to discuss the number of microspheres needed to attain statistical precision in microsphere measurements of blood flow, especially in smaller organs such as islets or organs with low blood perfusion. In a seminal paper in this context it was found that the number of microspheres within organs followed a Poisson distribution (113). By applying statistical calculations they determined that in order to obtain a 10% precision in their measurements, all samples, i.e. arterial reference sample and organ samples, should contain at least 384 microspheres (113). This topic has been an issue of much debate (149,150), and the consensus today is that the experimental error can be kept as low as 10%–15% also with a substantially lower number of microspheres in the samples. It was shown empirically in rats that this was the case when samples contained 200 microspheres (122). The application of the technique to ocular structures and the cochlea led to considerably lower numbers of microspheres in the tissue samples, but it was shown empirically and theoretically that the errors in the determinations still remained low (123,124) provided that the flow values exceeded 10 μL/min (125). In these instances as few microspheres as around 50 were sufficient.

When microsphere measurements of islet blood flow were initially performed, these issues were discussed in detail (71,83,87,126). When applying the microsphere technique in rats the reference sample contains >2,000 microspheres, whereas the whole pancreas contains 2,000 microspheres and the islets 200–400 depending on the experimental manipulations. Note that the microsphere content in all islets is summarized and the islets are considered to be one organ. The flow value to the whole islet organ is in the order of 60–100 μL/min, i.e. well within the limits to achieve results with statistical significance.

(v) Microspheres should be retained in the tissue. After injection the microspheres themselves are supposed to remain in the tissue, and not move after their initial impaction. Nevertheless, it might happen that microspheres leave the original organ of impact and move into the venous circulation, i.e. a delayed shunting. These microspheres usually end up in the lungs. By such means there is an underestimation of the true organ blood flow. In most cases when one blood flow measurement with microspheres is carried out, the organ is removed quickly (within minutes) after the injection, meaning that there is little time for any further movement to occur. However, if repeated measurements are performed there is an increased risk for this to occur. In the case that microspheres have different labels to enable separation of different batches, this at least makes it possible to compare this over time. Thus, the differently labeled microspheres within the lungs provide a measure of the degree of shunting of different microspheres (90). We have performed studies in rodents where we have injected three differently labeled microspheres (different colors) (90). We found no differences in basal blood flow and interpreted this to reflect that there is no real delayed shunting within the pancreas of rodents of 10 μm microspheres. These experiments also lend support to the notion that no circulatory artifacts are induced by 10 μm microspheres in the used doses, even if administration is repeated.

Another issue which may lead to delayed shunting is if the microspheres induce local ischemia in the tissue and thereby damage the vasculature so they can dislodge from their site of impaction. This has been described mainly for larger microspheres. In the myocardium up to 20% of the microspheres can escape, also through lymph (127,128). However, no signs of ischemia have been detected in the pancreas after microsphere injections in the doses we use in rodents.

Another, and potentially serious, source of error is the movement of microspheres within the organ, leading to redistribution with time between different compartments. Such a redistribution occurs in the intestine, where microspheres initially trapped in submucous capillary plexa can move to more distal mucosal capillaries (129,130). This is caused by diameter changes in the blood vessels ante- or post-mortem. In both these referred studies perfusion pressure was varied, e.g. by administration of isoproterenol. Obviously, it is difficult to use microspheres to quantitate the blood flow if capillary systems are connected in series with one another. In the liver, microspheres only allow for blood flow measurements in the

hepatic artery and not that through the portal vein (97). Similar difficulties are encountered also when measuring renal medullary blood flow, where the microspheres end up in glomeruli since they precede the medullar vessels (131).

Islet capillaries are in parallel to those of the exocrine pancreas so this is not a problem for islet blood flow measurements. However, the flow to the exocrine pancreas is underestimated by microspheres, since some islets are part of an insulo-acinar portal system (2), which means that pancreatic acini receiving their arterial blood from this system cannot be measured with microspheres. The exact contribution of the portal system to exocrine flow is unknown.

Extraction of microspheres within the tissue in proportion to blood flow assumes that the arterioles and capillaries are homogeneous with regard to diameter and do not contain any major thoroughfare channels with much larger diameter than the microspheres. If there are marked variations it may lead to preferential shunting in parts of the tissue, and thereby underestimations of flow. This is the case for many malignant tumors, which contain large and immature blood vessels, which often shunt microspheres (132,133). Islets have a homogeneous capillary diameter, but it is usually larger than that of exocrine capillaries (23), suggesting that islet microspheres could move from the islets into acinar capillaries or veins. Alternatively, microspheres in an afferent arteriole could potentially move from the arteriole, which is at least partially within exocrine tissue and into an islet. We have performed experiments where we have artificially altered the blood pressure, both *in vivo* within the autoregulatory capacity of islets (i.e. 70–140 mmHg) (38) and *in vitro* with different perfusion pressures (114), without observing any signs of this. Moreover, we have measured blood flow in islet adenomas in MEN1-knockout mice, but due to the considerations dealt with above we chose to use hydrogen gas clearance instead (29).

The possibility of delayed shunting of microspheres through an organ, in particular those with blood vessels with varying diameters, presents certain difficulties in the use of microspheres to assess organ blood flow. Thus, newly formed blood vessels in islet grafts are initially immature with wide diameter variations (26), leading to possible shunting or movements of microspheres. The use of other techniques such as laser-Doppler flowmetry (15) or hydrogen gas clearance (134) is therefore highly warranted.

(vi) Measuring accuracy. This is mainly an issue when tracers from the microspheres are extracted from the tissue of interest. Microspheres can be purchased labeled with a radioactive isotope, a fluorescent substance, or different colors (101). Radioactivity can be measured with conventional techniques, and as long as a whole organ is of interest there are usually no problems, provided that the isotope is stably incorporated into the microspheres. Fluorescence or dyes can be extracted from the organs of interest according to the manufacturer's instructions, usually involving basic liquids, and then quantified spectrophotometrically.

With regard to pancreatic islets there is an additional problem, which is that they constitute only 1%–2% of the pancreas. To quantify the number of microspheres within islets we mainly rely on direct microscopic counting of the microspheres. This can be done in histologic sections, but this is extremely cumbersome. It has, though, been tried on islet grafts (135). Since the microsphere diameter is fairly large, there is always a risk that the microtome damages them and thereby distorts the tissue architecture. When applying microspheres to islet blood flow measurements, different ways to clear the exocrine tissue (and thereby visualize both islets and microspheres in thick sections) have been applied. We have applied a freeze-thawing technique, which allows us to quantify microspheres and their localization with a high degree of certainty by manual counting (136,137). The islets can usually be identified directly as whitish aggregates of cells, and if stains such as neutral red or dithizone are applied, their identity can be confirmed (71,137). Alternatively, the pancreas can be cleared with e.g. methyl salicylate or other substances (71,87,138). It is important to ascertain that all microspheres have been visualized and that intra-islet microspheres are identified with certainty. The correlation between quantifications in cleared tissues and conventional histological sections is excellent (110).

An alternative approach would be to isolate the islets with collagenase and then count the number of microspheres being present in the islets, as well as in the remains of the digest. This has been tried, with radioactive microspheres, but the results have been published only in abstract form (107). The major problem with this approach is that only a limited number of islets can be isolated with this technique, usually in the order of 50%–60% of the larger islets, and none of the smaller ones. This means that only a sample of the islets can be used for the determinations, and since there is a heterogeneity in islet blood flow (*vide supra*) it is not known if this sample is representative. Fluorescent microspheres have been used, to isolate microsphere-containing islets, but in this context the exact degree of islet recovery is unknown (91,92).

(vii) Use of microspheres for other purposes than blood flow measurements. Microspheres can be impregnated with e.g. cytostatic drugs or radioactivity and then injected into organs where they become entrapped and then produce high local concentrations of drugs or radioactivity. This approach has especially been used to treat hepatic tumors since they receive almost all their blood from the hepatic artery. If tributaries to this artery are embolized with large microspheres—often sizes of up to 50 μm have been used—the normal liver parenchyma still receives sufficient oxygen and nutrients from the portal vein to survive. Such microspheres can be made of starch or albumin, or, in the case of radioactivity, yttrium-labeled polystyrene (139). We have tentatively tried this approach on islets implanted into the liver of mice. Such derive their blood from the hepatic artery (140), and we envisaged affecting islet graft cell replication. However, this did not meet with any success (unpublished observation). Since islet blood flow in the endogenous pancreas is much higher than that to the exocrine pancreas, such an approach would also be feasible for endogenous islets.

B. In vivo microscopy and pancreatic islet blood flow

By direct microscopic observations of islets, the earliest published in 1882 by Kühne and Lea, it has been possible not

only to map the vascular anatomy of the islets as outlined above, but also to make direct observations on microcirculation in islet capillaries (15,21,37,100). Confocal microscopy is pivotal to the recent advances in this field, which allows for visualization of the vascular architecture in thick sections and whole islets. Furthermore, improved possibilities to stain islet endothelial cells with different fluorescent antibodies and lectins have contributed to the increased use of these techniques (141). There are also transgenic mice whose islet endothelial cells are spontaneously fluorescent (46).

One disadvantage with these techniques has been that they are semi-quantitative. Possible ways to measure flow include laser-Doppler velocimetry, which uses the frequency shift produced by the Doppler effect when laser light is reflected from moving blood cells (142). The direct calibration of these values to blood flow is usually difficult to obtain, whereas relative changes in flow can be easily monitored (143). Another source of error when applying this technique is that the penetration depth of the laser light into the tissue needs to be evaluated, since blood cell movements in underlying tissues may otherwise disturb the measurements. So far this technique has been applied mainly to transplanted pancreatic islets (15,100,144), since most of the laser probes are too large to place over individual islets without undue disturbances from surrounding tissues.

Laser speckle is a random interference effect that gives a grainy appearance to objects illuminated by laser light. If the object consists of individual moving scatterers, such as blood cells, the speckle pattern fluctuates. These fluctuations provide information about the velocity distribution of the scatterers, i.e. blood cells (145). With the speckle technique it is also possible to devise a full-field technique that gives an instantaneous map of velocities in real time. This technique has recently been applied to transplanted islets (146), but its full potential has not yet been developed.

Another way to assess microvascular flow is the dual-slit cross-correlation technique (147), measuring the time it takes for a red blood cell to traverse from a pre-set point to another, which has been applied to islet grafts (10,100).

The techniques referred to above are all excellent for evaluations of graft blood flow or in some cases determinations of microcirculation in single islets. The great disadvantage is that they do not provide blood flow measurements per gram of organ weight for the whole islet organ. Because there are heterogeneities in islet blood flow, single islet flow determination becomes sensitive to such random variations.

A more recent application where the use of *in vivo* microscopy has been taken one step further is the migration of macrophages and polymorphic neutrophilic granulocytes (PMN) into islets. This has been applied both to transplanted islets and endogenous islets by Phillipson and co-workers, and they have been able convincingly to demonstrate that maintenance of normal islet vasculature and presumably β-cell mass and development depend on this (148–150). In view of the importance of inflammatory reactions in both T1D and T2D the true potential of this technique in this context is really staggering. Further details on the use of *in vivo* microscopy can be obtained elsewhere (21,46,100,148,151).

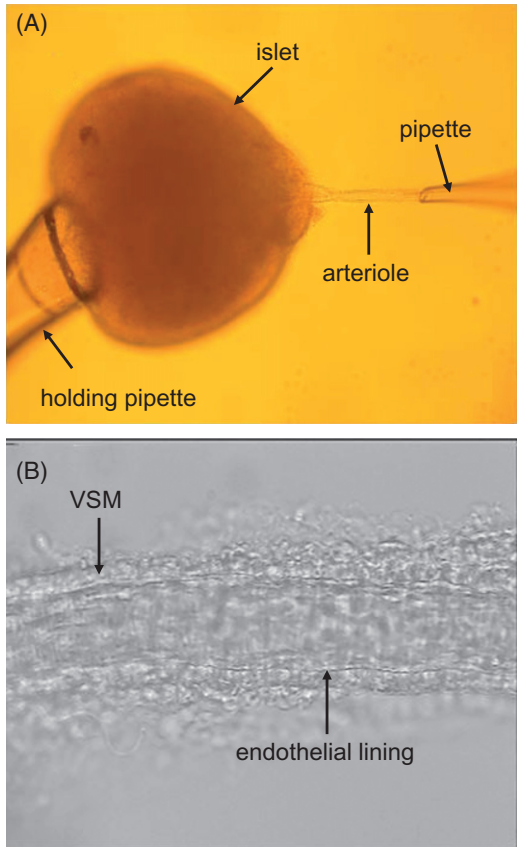

Figure 4. A: Pancreatic islet being perfused after cannulation with a glass pipette. The islet has a diameter of 250 μm. B: A close-up of the arteriole showing two rows of vascular smooth muscle. The diameter of the arteriole is 34 μm.

C. Perfusion of isolated, single islets

This technique is based on microdissection of single islets, a technique originally developed by Claes Hellerström (152), with their arteriolar supply intact. The islet can then be stabilized with holding pipettes, while the arteriole is cannulated with a custom-made glass pipette (30) as previously described for renal glomeruli (153). Islets are placed in a chamber on a stage of an inverted microscope where movement and adjustment of concentric holding and perfusion pipettes can be made (Figure 4A). The system is then perfused with buffer through the arteriole, and perifused around the islets, at a perfusion pressure of approximately 40 mmHg. The experimental set-up allows clear visualization of the arteriole, with its vascular smooth muscle, and measurements of contraction or dilation of the blood vessel (Figure 4B). This enables direct studies of the vascular reactivity of the arterioles, without any confounding factors from other tissues. However, although blood flow regulation of individual islets can be investigated in a unique manner, it is not possible to perform any measurements on islet blood flow.

D. Flow deposition techniques for studies of islet blood flow

These techniques are based on administration of substances which are deposited in a tissue or volume of tissue in proportion to its blood flow. This necessitates the choice of appropriate substances, as well as the possibilities to detect these

substances in the tissues of interest. A major difficulty is often the detection step, especially when small structures such as endogenous islets are of interest. However, the detection is non-invasive, which would be a major advantage. Another possible source of error is the degree of extraction of the tracer. If that is low, it means that the fractional extraction is higher in organs with low flow. This was the case for the initially used tracers such as rubidium-86 and potassium (131,154). Different substances labeled with technetium-99 (155) or xenon-133 (156) have been used as well. At least in their present form the resolution of the detectors applied with these techniques is not sufficient to resolve islet blood flow.

One more possibility, which so far has not been used for determinations of islet blood flow, is determinations of ^{14}C-antipyrine or ^{125}I-antipyrine in tissues. These substances accumulate in cells in relation to their delivery through blood and can then be determined autoradiographically. The resolution is excellent, but only relative values of blood flow are provided. Furthermore, the technique is very time-consuming and labor-intense (116,157).

We recently adapted the hydrogen gas clearance technique to measure both endogenous and transplanted islet blood flow (134). This gives the possibility accurately and repeatedly to measure the blood flow to the same islet. However, in order to perform the measurements thin platinum-electrodes must be inserted into the islets. These electrodes detect the hydrogen concentration in the tissue, and we can then measure its outflow after loading the animal with inhaled hydrogen. The outflow rate has been shown to be proportional to the blood flow. The requirements of electrode insertion limit the number of islets available for the measurements to those that can be directly observed. To increase this number we used duct-ligated rats. Ligation of the pancreatic duct leads to exocrine atrophy, but does not interfere with islet blood flow (158). These studies verified the actual blood flow values obtained with other techniques in endogenous and transplanted islets, and confirmed the heterogeneity of islet blood flow (134).

E. Radiological techniques to measure islet blood flow

Recent rapid advancements in MRI and PET techniques make it likely that it will soon be possible to measure blood flow in endogenous islets in humans. Indeed, a recent study from Turku has demonstrated that some conclusions on islet blood flow may be inferred from studies on pancreatic perfusion (159,160). Most studies are variants of flow deposit techniques, where different tracers have been applied, and conclusions on islet blood perfusion are indirect. There has been intense activity to identify optimal markers, and at present there are several available, some based on serotonin (161,162). Another potential application is the use of ^{18}F-fluoro-2-deoxy-D-glucose as a marker of inflammation. Nuutila and co-workers have shown that this substance is taken up in islets in NOD mice (163) and also that this substance can be used to detect inflammations in e.g. endocarditis (164). This substance would then serve as an excellent marker for insulitis, and such preliminary studies have been presented. In a recent study on LADA patients scintigraphy utilizing interleukin-2 radiolabeled with (99m)Tc ((99m)Tc-IL-2) was applied to detect insulitis (165), a technique previously used to image chronic inflammatory-mediated disorders.

4. Concluding remarks

Despite technical difficulties it is possible to determine islet blood flow in experimental animals. However, at present there is no method which allows for determinations of islet blood flow in humans. The knowledge gained so far from blood flow measurements in experimental animals has shown that the islets possess a blood flow regulation which is independent of that for the whole pancreas, and uniquely adapted to the metabolic needs of the endocrine cells. The pancreatic islet blood flow is 5–10 times higher than that of the exocrine pancreas, and can be selectively enhanced whenever the need for insulin secretion is increased. There is also compelling evidence that islet blood perfusion is disturbed during conditions with impaired glucose tolerance and overt type 2 diabetes. At present much work is put into efforts to determine whether blood perfusion disturbances are of pathogenic importance for the development of diabetes, or whether it is secondary to the metabolic disturbances within and outside of the islets.

Disclosure statement

The authors report no conflicts of interest.

Funding information

Own work referred to in the review has been supported by grants from the Swedish Research Council, the Swedish Diabetes Foundation, the Swedish Juvenile Diabetes Foundation, Diabetes Wellness Sverige, the Novo Nordisk Foundation, the Juvenile Diabetes Research Foundation, and the Family Ernfors Fund.

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