Short-term glucosamine infusion increases islet blood flow in anesthetized rats

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Impaired glucose tolerance and type 2 diabetes in rodents are associated with increased islet blood flow. If this is important for modulation of the endocrine function is at present unknown. We evaluated if glucosamine infusion, which induces peripheral insulin resistance and glucose intolerance, could be used to acutely increase islet blood flow. We infused anaesthetized Sprague-Dawley rats for 2 h with glucosamine (6 mg/kg body weight), in some cases followed by glucose administration. The former induced a 2-fold increase in serum insulin concentrations while plasma glucose remained unchanged. In vitro an augmented insulin response to hyperglycemia and decreased insulin content in batch type islet incubations with glucosamine for 24 h were seen. After 2 h glucosamine exposure in vitro, insulin release was decreased. In vivo glucosamine infusion increased islet blood flow, without affecting other regional blood flow values. Glucose increased islet blood flow to the same extent in control and glucosamine-infused rats. When exposed to 10 mmol/L glucosamine arterioles of isolated perfused islets showed a 10% dilation of their vascular smooth muscle. Thus, application of this model leads to acute hyperinsulinemia in vivo but a decreased insulin release in vitro, which suggests that effects not located to β cells are responsible for the effects seen in vivo. An increased islet blood flow in previously healthy animals was also seen after glucose administration, which can be used to further dissect the importance of blood flow changes in islet function.

Introduction

Pancreatic islet blood flow is regulated separately from that of the exocrine portions of the pancreas and is also 5–10 times higher in the islets.[1] Normally islet blood perfusion reflects the needs imposed by islet cell metabolism and is coupled to insulin release.[1] However, in rodent models of impaired glucose tolerance and overt diabetes there is an increased islet blood flow.[2-6] The reasons for this are largely unknown, even though it is likely that the autonomic nervous system plays a crucial role.[1,7] It is also probable that the hyperperfusion of blood affects islet endothelial cells and changes their release of autacoids which may adversely affect the functions and growth of endocrine cells.[8]

In all rodent models referred to above, there is usually a primary β-cell defect associated with peripheral insulin resistance, which together contributes to the impaired glucose tolerance or diabetes.[9] Most models, besides 48 h glucose infusions,[5] are due to inherited defects, which take time to develop into overt disease. However, short-term infusion of glucosamine stimulates the hexosamine cycle in many tissues.[10] This impairs β-cell function[11-13] and also induces a pronounced peripheral insulin resistance especially in skeletal muscle.[14] This is seen also in previously healthy animals with normal glucose tolerance.

The aim of the present study was to evaluate if the glucosamine model was associated with an altered islet blood flow, and if this was due to effects locally in islet vasculature or secondary to altered glucose metabolism. We found that islet blood flow was increased and this could to a major extent be explained by a direct effect on islet arteriolar vascular smooth muscle. Thus, by applying this model a selective hyperperfusion of the islets can be induced, which can be used to further dissect the importance of short-term islet blood flow changes for endocrine function.

Results

All animals tolerated the 2 h glucosamine infusion without any signs of infirmity. No changes in hematocrit or mean arterial blood pressure were observed (**Table 1**).

Glucosamine-infusion did not affect plasma glucose concentrations in control rats or after exogenous glucose administration (**Fig. 1A**). However, serum insulin concentrations were increased after 2 h of glucosamine infusion in the control animals (**Fig. 1B**). After glucose administration there were no differences in the serum insulin response between saline- and glucosamine-infused rats (**Fig. 1B**).

After in-vitro exposure to 10 mmol/L glucosamine for 24 h before the insulin release experiment was performed, the insulin released from isolated islets were similar in both pre-treated and control islets during low (1.67 mmol/L) glucose concentrations

Table 1. Anaesthetized male Sprague-Dawley rats were infused with saline (4 ml/kg BW x h) or glucosamine (6 mg/kg BW x h dissolved in saline) for 120 min. At 117 min, the animals were injected IV with 1 ml 30% D-glucose or saline. Measurements were made with a microsphere technique at 120 min

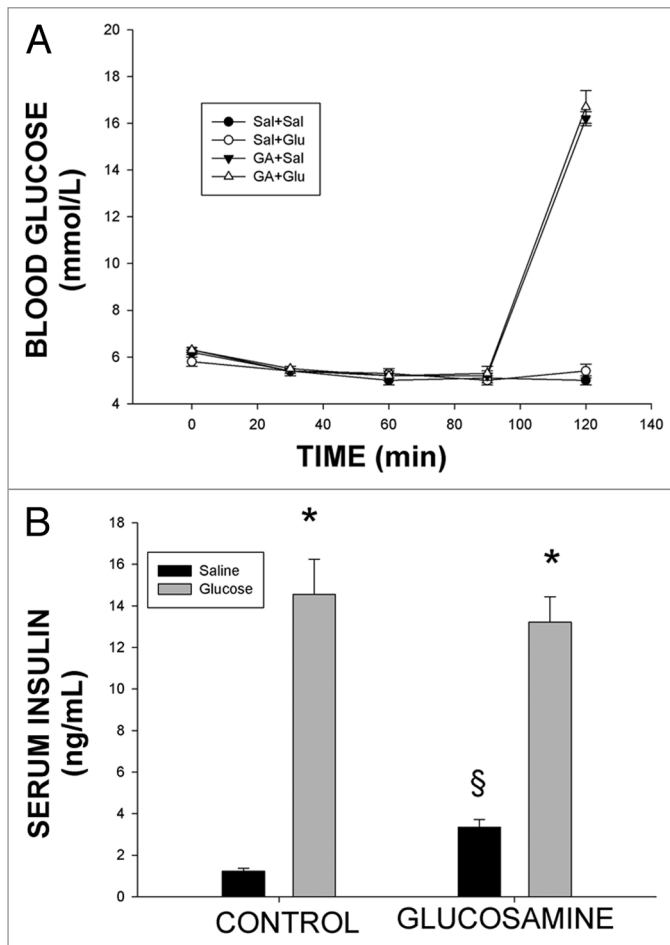| Treatment	Saline + saline	Glucosamine + saline	Saline + glucose	Glucosamine + glucose
No of animals	8	8	7	8
Hematocrit (%)	45.2 ± 0.5	45.2 ± 0.9	43.4 ± 0.8	44.0 ± 0.3
Mean arterial blood pressure (mmHg)	128 ± 4	128 ± 4	130 ± 4	133 ± 4
Duodenal blood flow (ml/min x g)	4.59 ± 0.40	4.48 ± 0.62	4.10 ± 0.62	4.64 ± 0.54
Colonic blood flow (ml/min x g)	0.99 ± 0.19	0.89 ± 0.14	0.89 ± 0.37	1.36 ± 0.38
Renal blood flow (ml/min x g)	5.84 ± 0.47	6.62 ± 0.96	5.06 ± 0.44	5.18 ± 0.94
Adrenal blood flow (ml/min x g)	4.38 ± 0.93	5.86 ± 0.72	4.71 ± 0.66	5.32 ± 0.92

All values are means ± SEM.

Figure 1. (**A**) Plasma glucose concentrations at different times in anaesthetized male Wistar-F rats infused with saline (Sal; 4 ml/kg body weight x h) or glucosamine (GA; 6 mg/kg body weight x h; dissolved in saline) for 120 min. At 117 min the animals were injected intravenously with 1 ml 30% D-glucose (Glu) or saline. (**B**) Serum insulin concentrations after the 2 h infusion in the same groups. Values are means ± SEM for 7–8 observations. * denotes $P < 0.001$ when compared with the corresponding saline-injected rats.

(**Fig. 2A**). However, the response to high (16.7 mmol/L) glucose was augmented after the 24 h glucosamine treatment (**Fig. 2A**). There was also a decrease in islet insulin content after 24 h treatment with glucosamine (**Fig. 2B**). When glucosamine was added only during the 2 h release experiments the insulin release at low glucose was higher than during 24 h of exposure (**Fig. 2A**). There was an increase in in glucose-stimulated insulin release in control islets, but a decrease in the islets with added glucosamine (**Fig. 2B**). No changes in insulin content were seen after 2 h glucosamine exposure (**Fig. 2B**).

Glucosamine infusion in control rats increased islet blood flow (**Fig. 3B**), but had no effect on total pancreatic blood flow (**Fig. 3A**), or duodenal, colonic, renal or adrenal blood flow (**Table 1**). As expected glucose increased islet blood flow in the control rats (**Fig. 3B**), but lacked effect on the other studied organ blood flow values (**Fig. 3A**; **Table 1**). Glucose administration to glucosamine-infused animals increased islet blood flow to the same extent as seen when compared with glucose-injected control rats (**Fig. 3B**). Also the other studied organ blood flow values were similar in glucose-injected rats irrespective of whether they were given saline or glucosamine (**Fig. 3A**; **Table 1**).

When the vascular reactivity in arterioles of isolated islets was examined 10 mmol/L of glucosamine caused a 10% dilation of the vascular smooth muscle (**Fig. 4**). All islet arterioles reacted with a pronounced vasoconstriction to KCl at the end of the experiments thereby confirming their viability.

Discussion

In the present study, we wanted to examine the effects of glucosamine on islet circulation with a view of obtaining a simple way of increasing islet blood flow, cf.[1] The rationale for this approach is previous suggestions that increased islet blood perfusion may adversely affect islet endocrine function.[1,15,16] Since hyperglycemia per se is one of the most potent stimulators of islet blood flow through interactions between local islet metabolism and the nervous system,[17] it would be advantageous to have access to a model more reminiscent of early impaired glucose tolerance characterized by e.g. insulin resistance and not overt hyperglycemia. By choosing a 2 h infusion of glucosamine, we could obtain such a condition with an acutely induced increase in serum insulin concentration and islet blood flow, with no effects on plasma glucose. If we had continued the infusion for 4 h or longer, as originally described[14,18] hyperglycemia also would have ensued.
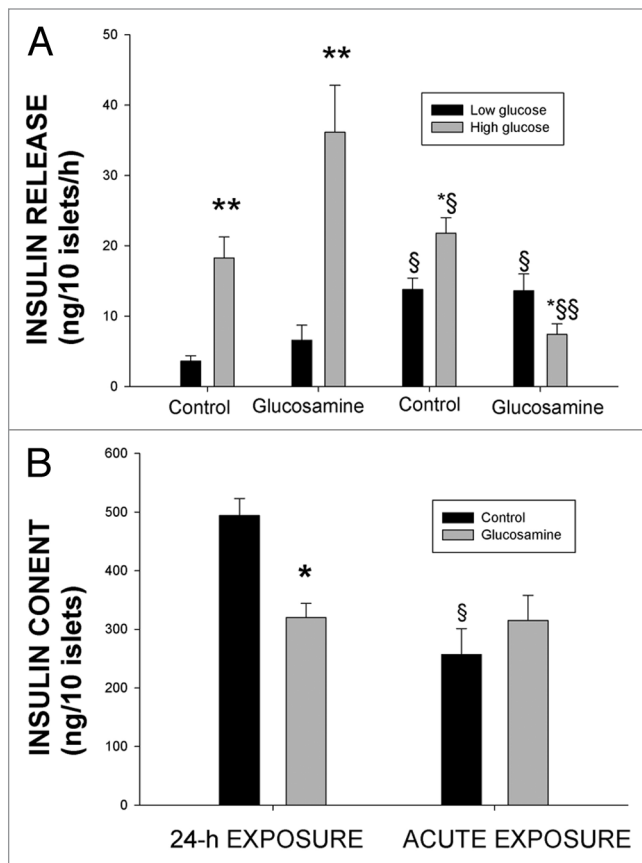
Figure 2. (**A**) Insulin release from isolated rat islets pre-cultured for 24 h with or without glucosamine (10 mmol/L; chronic exposure) or with gluosamine added only during the release experiments (acute exposure). During the release experiments the islets were incubated in Krebs-Ringer bicarbonate buffer with HEPES (N-[2-Hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid]) during 2 consecutive hours at 1.7 (low) and 16.7 (high) mmol/L glucose, respectively. (**B**) Insulin content of the same rat islets after the release experiments consisting of exposure to both low and high glucose concentrations. Values are means ± sem for 7–8 observations. * denotes $P < 0.05$ and ** $P < 0.001$ when compared with the corresponding experiments performed at low glucose concentrations and § denotes $P < 0.05$ and §§ $P < 0.01$ when compared with the corresponding value for chronic exposure.

We did indeed observe this, as well as an increased islet blood flow in pilot experiments.

The hexosamine biosynthetic pathway (HBP) is present in all cells in the body, its 2 major nutrient inputs being glucose and glucosamine.[19]

One important aspect of HBP is related to the fact that GlcNAcylation of the hydroxyl groups of serine and/or threonine residues of target proteins is an inducible, reversible, and dynamic posttranslational modification, which is involved in the regulation of multiple cellular processes.[20] Positive effects include protection against reperfusion injury[21] and improved organ function after trauma hemorrhage[22] and stress.[23] However, emerging evidence suggests that elevated GlcNAcylation of proteins contributes to glucose toxicity and is strongly associated with hyperglycemia-induced insulin resistance, 2 major hallmarks of type 2 diabetes.[20,24,25]

As referred to above glucosamine affects glucose metabolism due to its effects on GlcNAcylation. These effects are dose-dependent and the normal doses prescribed for osteoarthritis does not seem to impair glucose tolerance.[26] Interestingly, when higher doses are used the substance impairs β-cell function in humans[13] and in rodents.[12] In different animal models this has been suggested to be due to alteration of mitochondrial function.[11] It was recently also shown that murine β cells (and cell lines) have restricted actin organization upon glucosamine treatment by virtue of O-Glcnac modification of paxillin, which also interferes with insulin secretion.[27] We did indeed observe such effects when islets were acutely exposed for 2 h to glucosamine in vitro, but when they had been exposed for 24 h there was rather a potentiation of glucose-stimulated insulin release. This may well reflect an adaptation of the β cells to the effects induced by the glucosamine, and it should be noted that all islets were cultured for 3–4 d before the insulin release experiments which may affect their response. The reason why we did not see such effects in vivo in the present study despite the short time chosen for glucosamine infusion is difficult to ascertain. However, we interpret this, in combination with the flow data discussed below, to suggest a preferential acute effect of glucosamine of extra-pancreatic tissues in vivo. During longer treatment in vitro glucosamine is diabetogenic,[28] and mimics the β-cell dysfunction seen in type 2 diabetes.[29]

Augmented levels of O-GlcNAc in vasculature increases vascular reactivity to constrictor stimuli[30] and decreases endothelium-dependent vasodilation, which is associated with OGlcNAc modification of endothelial nitric oxide synthase.[30,31] In addition, augmented levels of O-GlcNAc have been found in the vasculature of mineralocorticoid hypertensive animals and may play a role on the vascular abnormalities in salt-sensitive hypertension.[32] This is in good accordance with findings in skeletal muscle where a 4 h infusion reduces skeletal muscle blood flow, measured by microspheres.[18] In later studies it has been shown that insulin resistance may occur due to impaired capillary recruitment in skeletal muscle.[33] On the other hand, Xing et al.[34] have suggested that O-GlcNAcylation may display cardioprotective and anti-inflammatory effects in arteries subjected to acute endoluminal injury. In most of these studies the exposure to HBP has been long, while the present study on isolated islet arterioles encompassed acute experiments. Interestingly a consistent and pronounced vasodilation was seen in islet arterioles when exposed to glucosamine concentration both in vivo and ex vivo. Interestingly, it has been observed that the effects of glucosamine was operative in normoglycemic, but not chronically hyperglycemic rats.[14] This confirms our findings that glucose administration did not affect blood flow in our study, or as previously observed when islet blood flow is already increased due to heightened functional demands after partial pancreatectomy.[35] The dilation was approximately 10% in the in vitro experiments, which can lead to a 50% increase in islet blood flow, since flow is proportional to the fourth power of the radius in a vessel. Thus, most of the islet blood flow increase seen after glucosamine administration in vivo can theoretically be explained by a local effect on the islet vasculature, and not
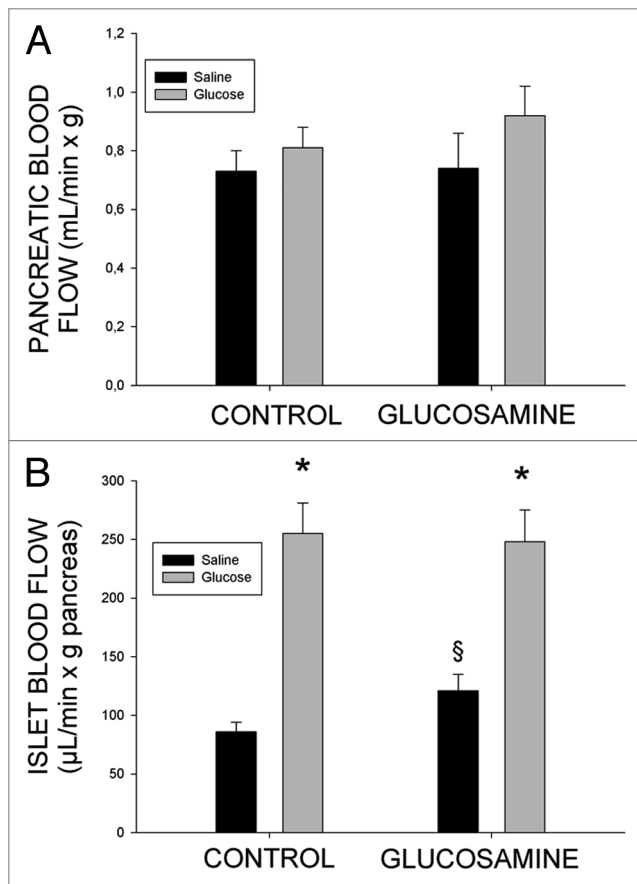
Figure 3. (**A**) Total pancreatic blood flow, (**B**) and islet blood flow in anaesthetized male Sprague-Dawley rats infused with saline (4 ml/kg body weight x h) or glucosamine (6 mg/kg body weight x h; dissolved in saline) for 120 min. At 117 min the animals were injected intravenously with 1 ml 30% D-glucose or saline. The values are means ± SEM for 7–8 experiments. * denotes $P < 0.05$ when compared with the corresponding saline-injected group. ¤ denotes $P < 0.05$ when compared with all other groups.

to changes secondary to altered glucose metabolism. In view of the findings in skeletal muscle referred to above, it may be that the HBP affects various blood vessels differently and may cause either vasoconstriction or vasodilation. Thus, the present study demonstrates that this model seems to present an islet blood flow increase preceding the development of β-cell impaired function. It would therefore open new ways to study the relationship between these events.

Materials and Methods

Animals

Male Sprague-Dawley rats (Scanbur) weighing 320 ± 4 g with free access to pelleted food and tap water were used in the experiments. All experiments were approved by the local animal ethics committee at Uppsala University.

Chemicals

Chemicals were purchased from Sigma-Aldrich unless given otherwise.

Islet isolation

Pancreatic islets were isolated from rats by collagenase digestion,[36] and cultured in groups of 150 islets for 3–4 d in 5 mL of culture medium consisting of RPMI 1640 supplemented with L-glutamine, benzylpenicillin (100 U/mL; Roche Diagnostics), streptomycin (0.1 mg/mL) and 10% (vol/vol) fetal calf serum. To some of the cultures glucosamine (10 mmol/L) was added for 24 h. Culture medium was changed every second day.

Glucose-stimulated insulin release and islet insulin contents

These procedures have been described in detail elsewhere.[36] After 3–4 d of culture groups of 10 islets, control or glucosamine pre-treated for 24 h, were transferred to vials containing Krebs-Ringer bicarbonate buffer supplemented with 10 mmol/L N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES), 3 mg/mL bovine serum albumin (BSA; ICN Biomedicals) and 10 mmol/L glucosamine; hereafter referred to as KRBH buffer. The KRBH buffer contained 1.67 mmol/L D-glucose during the first hour of incubation at 37° C (O_2/CO_2, 95:5). The medium was then removed and replaced by KRBH supplemented with 16.7 mmol/L glucose and the islets were then incubated for a second hour. After this second incubation the islets were harvested, following retrieval of the medium, and homogenized by sonication in 200 µL redistilled water. A fraction of the homogenate was mixed with acid-ethanol (0.18 mol/L HCl in 95% (vol/vol) ethanol) from which insulin was extracted overnight. Thus, these measurements were performed after exposure to both low and high glucose in all islets. Insulin contents in incubation media and homogenates were determined by a mouse insulin ELISA kit (Mercodia AB).

Blood flow measurements

This has been described in detail elsewhere.[37] The rats were anaesthetized with an intraperitoneal injection of thiobutabarbital sodium (120 mg/kg body weight; Inactin®; Research Biochemicals International). The animals were then placed on a heated operating table to maintain body temperature at approximately 37.5 °C. Polyethylene catheters were inserted into the ascending aorta, via the right carotid artery, and into the left femoral artery and vein. The former catheter was connected to a pressure transducer (PDCR 75/1; Druck Ltd), whereas the latter was used to infuse Ringer solution for 2 h (4 mL/kg body weight/h) with or without glucosamine (6 mg/kg body weight/h). After 117 min an intravenous injection of 1 mL saline or 30% (w/v) D-glucose was given. At 120 min blood flow values were measured as outlined below. A total of 3 x 10^5 black non-radioactive microspheres (EZ-Trac™; Triton Microspheres), with a diameter of 10 µm were injected via the catheter with its tip in the ascending aorta during 10 s.[37] Starting 5 s before the microsphere injection, and continuing for a total of 60 s, an arterial blood sample was collected by free flow from the catheter in the femoral artery at a rate of approximately 0.6 ml/min. The exact withdrawal rate was confirmed in each experiment by weighing the sample. Arterial blood was collected from the carotid catheter for determination of hematocrit, blood glucose concentrations with test reagent strips (MediSense AB) and serum insulin concentrations with ELISA (Rat Insulin ELISA™; Mercodia AB).
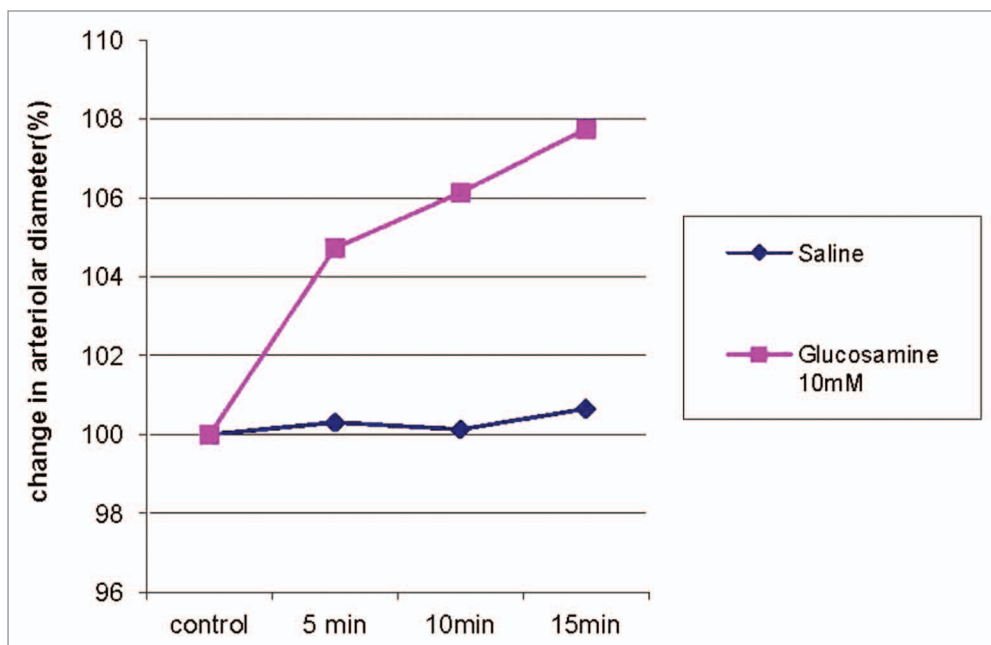
Figure 4. Changes in the diameter of islet arterioles isolated from normoglycemic Sprague-Dawley rats after administration of buffer containing 10 mmol/L glucosamine. Values are given in percent of the diameter before administration of the test substances (original diameter 22 ± 3 μm; n = 6). All values from 5 min and onwards are higher ($P < 0.05$) than in the control arterioles.

The animals were then killed and the pancreas and adrenal glands were removed in toto, blotted and weighed. Samples (approximately 100 mg) from the mid regions of the duodenum, descending colon and left kidney were also removed, blotted and weighed. The number of microspheres in the samples referred to above, including the pancreatic islets, was counted in a microscope equipped with both bright and dark field illumination after treating the organs with a freeze-thawing technique.[38] The number of microspheres in the arterial reference sample was determined by sonicating the blood, and then transferring samples to glass microfiber filters (pore size < 0.2 μm), and then counting them under a microscope.

The organ blood flow values were 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, N_{org} is number of microspheres present in the organ and N_{ref} is number of microspheres in the reference sample. With regard to islet blood perfusion it was expressed both per gram wet weight of the whole pancreas, and the estimated wet weight of the islets.

Blood flow values based on the microsphere contents of the adrenal glands were used to confirm that the microspheres were adequately mixed in the circulation. A difference < 10% in the blood flow values was taken to indicate sufficient mixing, and this occurred in all animals in the present study (data not shown).

Isolation and preparation of islets for single-islet perfusion

Rats were killed by an overdose of pentobarbital, and the pancreas was quickly removed and placed in cold (4 °C) albumin-enriched (1%) Dulbecco's Minimal Enriched medium (DMEM; Sigma-Aldrich). Islets were dissected with their arterioles intact[39] using a modification of a previously described technique for renal glomeruli.[40,41] The time for dissection was limited to 60 min and the obtained islets had diameters of approximately 200 μm. For further details, see our previous publications.[39,42] The experimental set-up allowed us to measure the diameter of the afferent islet arterioles continuously and to record changes at a resolution of < 0.2 μm.

Each experiment began with a 15 min equilibrium period with buffer containing 5.5 mmol/L glucose in both bath and perfusion solutions. Thereafter 10 mmol/L of glucosamine was added to the buffer and the islets were perfused with this for 15 min. Each perfusion was terminated by administration of KCl (100 mmol/L) to ascertain that the arterioles were able to contract.

Statistical calculations

All values are given as means ± SEM. Probabilities (P) of chance differences were calculated with Student's unpaired t test, or one-way repeated measurement ANOVA with the Tukey correction (SigmaStat™; SSPD). A value of $P < 0.05$ was considered to be statistically significant.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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