Early deficits in insulin secretion, beta cell mass and islet blood perfusion precede onset of autoimmune type 1 diabetes in BioBreeding rats

Anya Medina1, Saba Parween2, Sara Ullsten3, Neelanjan Vishnu1, Yuk Ting Siu1, My Quach3, Hedvig Bennet1, Alexander Balhuizen1, Lina Åkesson1, Nils Wierup1, Per Ola Carlsson3, Ulf Ahlgren2, Åke Lernmark1, Malin Fex1

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Abstract
Aims/hypothesis Genetic studies show coupling of genes affecting beta cell function to type 1 diabetes, but hitherto no studies on whether beta cell dysfunction could precede insulitis and clinical onset of type 1 diabetes are available.

Methods We used 40-day-old BioBreeding (BB) DRLyp/Lyp rats (a model of spontaneous autoimmune type 1 diabetes) and diabetes-resistant DRLyp/+ and DR+/+ littermates (controls) to investigate beta cell function in vivo, and insulin and glucagon secretion in vitro. Beta cell mass was assessed by optical projection tomography (OPT) and morphometry. Additionally, measurements of intra-islet blood flow were performed using microsphere injections. We also assessed immune cell infiltration, cytokine expression in islets (by immunohistochemistry and qPCR), as well as islet Glut2 expression and ATP/ADP ratio to determine effects on glucose uptake and metabolism in beta cells.

Results DRLyp/Lyp rats were normoglycaemic and without traces of immune cell infiltrates. However, IVGTTs revealed a significant decrease in the acute insulin response to glucose compared with control rats (1685.3 ± 121.3 vs 633.3 ± 148.7; p < 0.0001). In agreement, insulin secretion was severely perturbed in isolated islets, and both first- and second-phase insulin release were lowered compared with control rats, while glucagon secretion was similar in both groups. Interestingly, after 5–7 days of culture of islets from DRLyp/Lyp rats in normal media, glucose-stimulated insulin secretion (GSIS) was improved; although, a significant decrease in GSIS was still evident compared with islets from control rats at this time (7393.9 ± 1593.7 vs 4416.8 ± 1230.5 pg islet−1 h−1; p < 0.0001). Compared with controls, OPT of whole pancreas from DRLyp/Lyp rats revealed significant reductions in medium (4.1 × 109 ± 9.5 × 107 vs 3.8 × 109 ± 5.8 × 107 μm3; p = 0.044) and small sized islets (1.6 × 109 ± 5.1 × 107 vs 1.4 × 109 ± 4.5 × 107 μm3; p = 0.035). Finally, we found lower intra-islet blood perfusion in vivo (113.1 ± 16.8 vs 76.9 ± 11.8 μl min−1 [g pancreas]−1; p = 0.023) and alterations in the beta cell ATP/ADP ratio in DRLyp/Lyp rats vs control rats.

Conclusions/interpretation The present study identifies a deterioration of beta cell function and mass, and intra-islet blood flow that precedes insulitis and diabetes development in animals prone to autoimmune type 1 diabetes. These underlying changes in islet function may be previously unrecognised factors of importance in type 1 diabetes development.

Keywords Beta cell dysfunction · Beta cell mass · Insulin secretion · Islet blood flow · Type 1 diabetes

Saba Parween and Sara Ullsten contributed equally to this work.

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© Anya Medina
anya_medina.benavente@med.lu.se
1 Lund University Diabetes Centre, Clinical Research Centre, Skåne University Hospital (SUS), Jan Waldentrömstgata 35, SE-20502 Malmö, Sweden

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1 Umeå Centre for Molecular Medicine, Umeå University, Umeå, Sweden
3 Medical Cell Biology, Uppsala Biomedical Centre, Uppsala, Sweden

© Anya Medina
anya_medina.benavente@med.lu.se
Introduction

Type 1 diabetes is associated with the immune-mediated destruction of islet beta cells. Studies in human monozygotic twins, sharing identical genomes, demonstrate pairwise type 1 diabetes of 13–52%, suggesting that environmental and genetic causes may contribute similarly to the disease [1].

Research pertaining to the genetic contribution of type 1 diabetes have for the past decades focused on genetic loci implicated in regulation and selection of autoreactive T lymphocytes [2], although single nucleotide polymorphisms within the human insulin (INS) gene (mainly present in beta cells) remain one of the most important risk factors for the development of type 1 diabetes [3]. Recent studies have revealed that several candidate genes found in genome-wide association studies of type 1 diabetes susceptibility loci are expressed in beta cells and could thus influence beta cell function [4].

The BioBreeding (BB; LEW.1WR1) rat acts as a model of type 1 diabetes, whereby type 1 diabetes is suggested to originate from selective autoimmune destruction of beta cells [5]. As in humans, the major histocompatibility complex holds genetic factors that predict disease in this model [6, 7]. This explains some, but not all, of the inherited predisposition to type 1 diabetes. In the inbred BB rat strain BBDR Lyp/Lyp (herein referred to as DRLyp/Lyp), onset of type 1 diabetes is linked to lymphopaenia, which is caused by a frameshift mutation in the Gimap5 gene, while their littermates DRLyp/+ and DR+/- are resistant to diabetes [8, 9]. Loss of T cells because of lymphopaenia affects both CD4+ and CD8+ T cells, especially ART2.1+ T cells [5]. In fact, depletion of the ART2.1+ T cells in diabetes-resistant BB rats induces type 1 diabetes, suggesting that loss of regulatory T cells is associated with insulin and type 1 diabetes [10].

Early changes in beta cell function and blood glucose have not been elucidated in DRLyp/Lyp rats, although local changes in beta cells in inbred DRLyp/Lyp are reflected by production of eotaxin (an eosinophil and mast cell recruiting factor) in islets at about 40 days of age, before insulitis, hyperglycaemia and type 1 diabetes [11, 12]. However, positive staining of infiltrating monocytes remains to be shown at this age [11]. Additionally, islets from 40-day-old DRLyp/Lyp animals express lower levels of genes involved in the metabolism of reactive oxygen species (ROS) [13] and are more sensitive to changes in redox balance [14]. Over time, such an inherent sensitivity could contribute to accumulation of the ROS that diminish beta cell function, rendering cells more sensitive to immune cell attack.

Islet function is also dependent on functional islet vasculature and blood flow. In fact, inflammatory changes in vascular endothelial cells, characterised by increased expression of surface receptors, facilitate immune cell extravasation into the inflamed tissue [15]. Additionally, islet vasculature plays a critical role in maintaining oxygen and nutrient supply to the islets [16] and poor intra-islet blood flow is associated with changes in acute insulin response to glucose in vivo [17]. Interestingly, venular defects were observed in islets from BB (DP-BB/Wor) rats [18]. This, in combination with an underlying beta cell defect, could impair beta cell function and promote insulitis and beta cell destruction.

Currently, evidence of changes in beta cell function prior to onset of type 1 diabetes is limited. Therefore, we set out to explore whether insufficient beta cell function, or changes in beta cell mass and intra-islet blood flow, precede type 1 diabetes using the DRLyp/Lyp rat as a disease model.

Methods

Animals The BB rat was originally derived from a Canadian colony of outbred Wistar rats (originating from the Ottawa Health Research Institute, University of Ottawa, Ottawa, ON, Canada) that spontaneously develop hyperglycaemia and ketoadeosis, characteristics of clinical onset of type 1 diabetes. Heterozygous BB DRLyp/+ rats were used to obtain congenic DRLyp/Lyp rats as previously described [9, 19]. Briefly, the Lyp region from diabetes-prone BB rats was introgressed onto the diabetes-resistant BB rat and kept in sibling breeding for more than 50 generations by heterozygous breeders to yield 25% DRLyp/Lyp, 25% DR+/- and 50% DRLyp/+ rats. All DRLyp/Lyp rats developed diabetes after transferring the entire colony from University of Washington, Seattle to Lund University (including the Clinical Research Centre in Malmö, Sweden), in 2008. Animals were bred/kept in a pathogen-free environment at the Clinical Research Centre in Malmö, Sweden. They were housed at 21–23°C (12 h light/dark cycle) and fed ad libidum. All experiments were approved by the Animal Ethical Committee in Uppsala and Lund. All animals used in experiments were 40 days old unless otherwise stated.

Genotyping Tail snips were obtained from rat pups between 25–30 days of age. DNA was isolated and genotyped based on microsatellite analysis, as previously described [9, 20].

Blood glucose and plasma insulin levels Blood glucose was tested daily at 08:00 hours in DRLyp/Lyp (n = 225, 129 male [M]/96 female [F]) and control rats (DRLyp/+ and DR+/-;
n = 100, 50M/50F) from day 37 (ELTE XL glucometer; Bayer Diabetes Care, Tarrytown, NY, USA). Animals were considered to have developed diabetes when blood glucose levels were >11.1 mmol/l for two consecutive days. Serum insulin was measured in a baseline group at 37–41 days of age (DRLyp/Lyp: n = 7, 4M/3F; control rats: n = 10, 5M/5F), at 50 days (DRLyp/Lyp: n = 6, 3M/3F; control rats: n = 10, 5M/5F), at 60 days (DRLyp/Lyp: n = 6, 3M/3F; control rats: n = 11, 6M/5F) and at type 1 diabetes onset (DRLyp/Lyp: n = 7, 4M/3F; control rats: n = 9, 5M/4F) in 10 μl of serum (rat insulin ELISA, Mercodia, Uppsala, Sweden). Blood was obtained from venipuncture of the tail vein in the fed state.

IVGTT Glucose (1 g/kg) (Sigma Aldrich, Stockholm, Sweden) was injected into the tail vein of DR Lyp/Lyp (n = 10, 6M/4F) and control (n = 10, 6M/4F) rats after 6 h of fasting. Blood samples were collected from the sublingual vein at 0, 1, 5, 10, 20, 50 and 75 min. Plasma glucose and insulin levels were measured (Infinity Glucose Oxidase Liquid Stable Reagent, Thermo Scientific, Waltham, MA, USA and Rat Insulin measurement (Infinity Glucose Oxidase Liquid Stable Reagent, Thermo Scientific, Waltham, MA, USA and Rat Insulin ELISA, Mercodia). Plasma glucose and insulin samples were collected from the sublingual vein at 0, 1, 5, 10, 20, 50 and 75 min. Plasma glucose and insulin levels were measured (Infinity Glucose Oxidase Liquid Stable Reagent, Thermo Scientific, Waltham, MA, USA and Rat Insulin ELISA, Mercodia).

Perfusion of isolated islets Islets from DRLyp/Lyp (n = 14, 9M/5F) and control (n = 8, 4M/4F) were isolated using collagenase digestion and incubated in RPMI-1640 medium containing 11.1 mmol/l glucose (Sigma Aldrich) + 10% FBS overnight at 37°C. Seventy islets per chamber were used in perfusion experiments (Suprafusion 1000 System; Brandel, Glasgow, UK). Islets were perfused with secretion assay buffer (SAB) containing: 114 mmol/l NaCl, 4.7 mmol/l KCl, 1.2 mmol/l KH₂PO₄, 1.16 mmol/l MgSO₄, 25.5 mmol/l NaHCO₃, 20 mmol/l HEPES, 2.5 mmol/l CaCl₂ and 0.2% BSA (fatty acid free) (pH 7.2), supplemented with 2.8 mmol/l glucose for 2 h prior to sampling. Consecutive samples were taken at 2.8 mmol/l glucose to determine basal insulin release before challenging islets with a high glucose concentration (16.7 mmol/l). Experiments were concluded by estimating maximal insulin response by the addition of SAB containing 35 mmol/l KCl. The flow rate was 0.1 ml/min and temperature was kept at 37°C. Each fraction of perfusate was collected at 4 min intervals and stored at −20°C until analysed (Rat Insulin ELISA, Mercodia).

Batch incubation of isolated islets of Langerhans Isolated islets from DRLyp/Lyp and control rats were cultured overnight (RPMI-1640 medium, 11.1 mmol/l glucose, 10% FBS [Sigma Aldrich]; DRLyp/Lyp: n = 6, 3M/3F; controls: n = 6, 3M/3F), or for 5–7 days (RPMI medium, 5.6 mmol/l glucose, 10% FBS + penicillin [100 units/ml]–streptomycin [100 μg/ml]; DRLyp/Lyp: n = 6, 3M/3F; controls: n = 7, 3M/4F) at 37°C, 5% CO₂. Groups of three islets were placed in a well of a 96-well plate with SAB containing either 2.8 mmol/l or 16.7 mmol/l glucose at 37°C, 5% CO₂. Experiments were performed with 6–8 replicates for each condition. Insulin and glucagon levels were determined after 1 h (Rat Insulin ELISA and Glucagon ELISA, respectively; Mercodia).

Insulin content Total insulin was extracted from 50 islets per animal (DRLyp/Lyp: n = 6, 3M/3F; controls: n = 6, 3M/3F) using acid ethanol (0.18 mmol/l HCl in 95% ethanol). Extracted insulin was diluted and total insulin was measured (Rat Insulin ELISA; Mercodia).

qPCR of islets of Langerhans Isolated islets from DRLyp/Lyp (n = 6, 3M/3F) and control (n = 7, 3M/4F) rats were frozen (−80°C) after isolation or after 5–7 days in culture (37°C, 5% CO₂ in RPMI medium, 5.6 mmol/l glucose, 10% FBS + penicillin [100 units/ml]–streptomycin [100 μg/ml]). Total RNA was extracted (RNAeasy RNA purification kit; Qiagen, Hilden, Germany) and equal quantities of RNA were reverse transcribed (RevertAid First-Strand cDNA synthesis kit; Fermentas, Vilnius, Lithuania). mRNA levels were quantified (Maxima Probe/ROX qPCR Master Mix; Fermentas, Thermo Scientific, Helsingborg, Sweden) using an ABI PRISM 7900 (Applied Biosystems ViiA Real Time PCR System; Life Technologies, Foster City, CA, USA), using probes for Ilnb (ID no. Rn00580432), Tnf-α (also known as Tnfa) (ID no. Rn01525859), Ifng (ID no. Rn00594078) and Glut2 (also known as Slc2a2) (ID no. Rn00563565) (Applied Biosystems). Samples were run in triplicate and the transcript quantity was normalised to the geometric mean of mRNA levels of the reference genes (Applied Biosystems) Ppia (ID no. Rn00690933), Polr2a (ID no. Rn01752026) and Hprt (also known as Hprt1) (ID no. Rn01527840), using the formula 2^[(minCt – sampleCt)].

Blood flow measurements and islet morphometry DRLyp/Lyp (n = 11, 4M/7F) and control (n = 15, 6M/9F) rats were anaesthetised (i.p. injection of thiobutabarbital sodium; 120 mg/kg; Inactin; Sigma Aldrich) and placed on a heating pad to maintain body temperature. The trachea was detached and a polyethylene catheter was inserted to secure free airways. Catheters were inserted into the right ascending aorta and the left femoral artery. A pressure transducer was connected to the ascending aorta catheter. A blood sample was taken for blood glucose measurement (Freestyle Lite; Abbott, Calameda, CA, USA). When blood pressure had stabilised (10–15 min), animals were injected with 1.5 × 10⁵ microspheres (diameter: 10 μm) (E-Z Trac Ultraspheres; Stason Labs, Irwin, CA, USA) into the ascending aorta and blood was collected as described [21]. Animals were then euthanised and the pancreas and adrenal glands were dissected, weighed, cut in pieces and placed between object glasses. Object glasses containing
pancreatic tissue were freeze-thawed to visualise islets [21]. The percentage of islet volume was determined by a point-counting [22], and the number of microspheres in the exocrine and endocrine pancreas, adrenal glands and reference sample was counted in a bright and dark field illumination microscope.

Optical projection tomography imaging and quantification of islet beta cell distribution Following euthanisation using CO₂, pancreases from DR/Lyp/Lyp (n = 6, 4M/2F) and control (n = 4, 2M/2F) rats were excised and processed for optical projection tomography (OPT) imaging [23]. Antibodies used for whole mount immunohistochemistry were: guinea pig anti-insulin (1:500; A0564; DAKO Denmark, Glostrup, Denmark) and IRDye 680 goat anti-guinea pig (1:250; 926-68077; LI-COR Biosciences, Lincoln, NE, USA). Pancreatic lobes were scanned individually using a near-infrared OPT setup equipped with a 665/45 excitation and a 725/50 emission filter (Chroma). Beta cell volumes were reconstructed based on the signal from insulin-specific antibodies and pseudo-coloured to highlight the distribution of small <1 × 10⁶ μm³ (white), medium 1 × 10⁶ μm³ to 5 × 10⁶ μm³ (yellow) and large >5 × 10⁶ μm³ (red) islets [23, 24].

Live single cell ATP/ADP ratio measurements Single cell ATP/ADP ratio measurements in islets from DR/Lyp/Lyp (n = 91 islets) and control rats (n = 70 islets) were performed using the ATP biosensor, Perceval (Addgene, Cambridge, MA, USA). Islets were transduced [25, 26], plated and incubated on poly-D-lysine coated 8-well chambered cover glasses (Thermo Scientific, Waltham, MA, USA) for 2 h with RPMI medium containing penicillin (100 units/ml)–streptomycin (100 μg/ml) containing the Perceval adenovirus. Fresh medium was added and cells were incubated overnight. The following day, cells were pre-incubated at 37°C in 400 μl buffer P (135 mmol/l NaCl, 3.6 mmol/l KCl, 1.5 mmol/l CaCl₂, 0.5 mmol/l MgSO₄, 0.5 mmol/l Na₂HPO₄, 10 mmol/l HEPES, 5 mmol/l NaHCO₃, pH 7.4) containing 2.8 mmol/l glucose for 1.5 h. After this, cells were first imaged in the presence of low (2.8 mmol/l) glucose and then in the presence of high glucose (16.7 mmol/l) to investigate the basal and stimulated ATP/ADP ratio. Thereafter, ATP synthesis was inhibited by the addition of the ATP synthase inhibitor oligomycin (0.02 mg/ml) and an ionophore that uncouples ATP synthesis, carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP; 0.05 mmol/l). Cells were imaged using 490 nm excitation and 520 nm emission filter settings on a Zeiss LSM510 inverted confocal fluorescence microscope (Zeiss, Oberkochen, Germany).

Immunohistochemical analysis of islets of Langerhans Pancreatic sections from DR/Lyp/Lyp (n = 10, 5M/5F) and control (n = 10, 5M/5F) rats were collected on slides and air-dried overnight at 37°C. Slides were deparaffinised [27] and sections incubated with the following primary antibodies overnight at 4°C in moisturising chambers: mouse anti-glucagon (1:9000; G-2654, Sigma Aldrich), guinea pig antiproinsulin (1:2500; 9003; EuroDiagnostica) and rabbit anti-CD3 (1:200; C7930; Sigma Aldrich). Sections were rinsed in PBS with Triton X-100 for 2 × 10 min. Antibodies for insulin and glucagon was carefully validated as detailed [27, 28]. CD3 specificity was tested using primary antisera pre-absorbed with homologous antigen (100 μg/ml antiserum). Pancreatic sections were incubated with the following secondary antibodies with specificity for mouse, guinea pig, or rabbit IgG: goat anti-mouse Alexa Fluor 568, (1:400; A21124; Invitrogen, Thermo Scientific, Helsingborg, Sweden), goat anti-guinea Pig, Alexa Fluor 594, (1:400; A11076; Thermo Scientific) and goat anti-rabbit, Alexa Fluor 594, (1:400; A11012; Thermo Scientific) [27].

Immunofluorescence was examined in an epifluorescence microscope (Olympus, BX60, Tokyo, Japan). By changing filters, double staining was used to determine the location of the different secondary antibodies in one sample. Images were captured with a digital camera (Nikon DS-2Mv, Tokyo, Japan).

Statistical analysis Data are expressed as mean ± SEM. IVGTTs, AUC and acute insulin response to glucose (AIRglucose) were calculated as described [6, 29, 30]. Mann–Whitney non-parametrical testing was employed in all experiments, except for analysis of islet size (OPT), blood flow measurements, 1 h batch experiments, insulin content, qPCR and ATP/ADP measurements, which were analysed with Student’s t tests, and plasma insulin levels, which were assessed using a two-way ANOVA. Statistical analyses were performed using GraphPad Prism 6 software (GraphPad Software, La Jolla, CA, USA). p < 0.05 was considered to be statistically significant. All experiments were performed and analysed in a randomised and blinded fashion when possible. Outliers were identified using Grubbs test for outliers.

Results Diagnosis of diabetes DR/Lyp/Lyp and control (DR/Lyp/+ and DR/+ +) rats were followed by daily blood glucose measurements until diagnosis of type 1 diabetes (Fig. 1a). Cumulative incidence revealed that all DR/Lyp/Lyp rats had developed diabetes by 80 days of age (Fig. 1b). Mean age at onset of type 1 diabetes was 60 days ranging from 47 to 80 days (Fig. 1d). Female rats developed diabetes earlier than males (Fig. 1c; p = 0.004).

Serum insulin prior to type 1 diabetes onset Basal insulin levels were evaluated in DR/Lyp/Lyp and control rats over time. Despite normoglycaemia prior to onset of type 1
diabetes, insulin levels were lower at all time points in DRLyp/Lyp rats and failed to increase with age compared with control rats (Fig. 2a; \( p = 0.0004 \)).

**In vivo insulin release is perturbed in DRLyp/Lyp rats** In vivo glucose homeostasis and beta cell function were assessed with an IVGTT in DRLyp/Lyp rats. DRLyp/Lyp rats remained glucose tolerant (Fig. 2b). No difference in glucose clearance between groups was observed, also shown as AUC for glucose (Fig. 2d). However, DRLyp/Lyp rats secreted less insulin during the initial time points of the IVGTT vs controls (Fig. 2c) which was further highlighted by a reduction in AUC for insulin in DRLyp/Lyp rats (Fig. 2e; 19466.9 ± 1060.2 vs 14310.8 ± 1454.2 pmol/l × min; \( p = 0.04 \)) and a decrease in the AIRGlucose (Fig. 2f; 1685.3 ± 121.3 vs 633.3 ± 148.7; \( p < 0.0001 \)).

**Insulin secretion is decreased in islets from DRLyp/Lyp rats** To assess differences in insulin release (as evident by the IVGTT) between DRLyp/Lyp and control rats, we characterised the dynamics of insulin secretion in vitro using a perfusion setup. Islets from DRLyp/Lyp and control rats were first subjected to a low concentration of glucose (2.8 mmol/l) (Fig. 3a). Basal insulin secretion was similar between the groups. When challenging islets with a stimulatory concentration of glucose (16.7 mmol/l) during a 40 min period, the amount of insulin secreted by islets from DRLyp/Lyp rats was reduced. Control rats responded robustly to elevated glucose concentrations (Fig. 3b; control vs DR/Lyp/Lyp AUC: 398.2 ± 53.8 vs 206.1 ± 21.6 pmol/l × min; \( p = 0.002 \)). When islets were further challenged with 35 mmol/l KCl and 16.7 mmol/l glucose for 12 min, islets from DRLyp/Lyp rats continued to secrete less insulin than those from control rats (Fig. 3c; control vs DR/Lyp/Lyp AUC: 171.5 ± 18.8 vs 123.9 ± 14.9 pmol/l × min; \( p = 0.02 \)). Insulin content, however, was similar in islets from DRLyp/Lyp and control rats (Fig. 3d).

Comparable results to those obtained in perfused islets were observed when islets were exposed to low (2.8 mmol/l) and high (16.7 mmol/l) glucose concentrations during a 1 h static incubation. A reduction both in basal insulin secretion (282.5 ± 59.4 vs 186.0 ± 62.3 pg islet\(^{-1}\) h\(^{-1}\); \( p = 0.003 \)) and in glucose-stimulated insulin secretion (GSIS; 963.1 ± 162.1 vs 280.3 ± 64.4 pg islet\(^{-1}\) h\(^{-1}\); \( p = 0.0001 \)) from islets from
DRLyp/Lyp rats vs control rats was evident (Fig. 3e). Glucagon secretion was similar in islets from both groups when exposed to low and high glucose concentrations (ESM Fig. 1a).

Previous work suggests that removing islets from an inflammatory milieu can restore GSIS [31]. Therefore, we cultured islets from DRLyp/Lyp and control rats for 5–7 days. Insulin secretion was measured after exposure to low (2.8 mmol/l) and high (16.7 mmol/l) glucose concentrations in a 1 h static incubation. Overall insulin secretion was improved, both in DRLyp/Lyp and control rat islets, but a significant decrease in GSIS was still evident in islets from DRLyp/Lyp rats vs controls (Fig. 3f; 4416.8 ± 1230.5 vs 7393.9 ± 1593.7 pg islet⁻¹ h⁻¹; \( p < 0.0001 \)).

**II1b, Ifng and Tnf-α expression in islets isolated from DRLyp/Lyp rats**

Next we determined expression of cytokines in islets isolated from DRLyp/Lyp and control rats. RNA was extracted either immediately after isolation or after culturing islets for 5–7 days. Il1b was present at similar levels in islets just after isolation (ESM Fig. 1b). However, Tnf-α and Ifng were undetectable. When islets where cultured over a 5–7 day period, detectable levels of all cytokines were present (ESM Fig. 1c) but did not differ between groups.

**Islet blood perfusion**

To determine if reduced insulin secretion in vivo was associated with microcirculatory changes [17,32], we measured islet blood perfusion. Mean arterial blood pressure was recorded in animals prior to blood flow measurements with no significant difference between the two groups (data not shown).

Whole pancreatic blood flow did not differ between DRLyp/Lyp and control rats (Fig. 4a). Interestingly, islet blood flow was significantly reduced by 25% in the DRLyp/Lyp animals vs controls (Fig. 4b; 76.9 ± 11.8 vs 113.1 ± 16.8 μl min⁻¹ [g pancreas]⁻¹; \( p = 0.023 \)).

**Small and medium sized islets are less common in the pancreas of DRLyp/Lyp rats**

To understand whether the observed perturbation in insulin secretion in vivo was accompanied by differences in beta cell mass, we performed OPT on the whole pancreas from DRLyp/Lyp and control rats. Overall, beta cell mass did not differ between groups (Fig. 5a). However, there was a reduction in small (1.4 × 10⁷ ± 4.5 × 10⁶ vs 1.6 × 10⁷ ± 5.1 × 10⁶ μm³; \( p = 0.035 \)) and medium sized islets (3.8 × 10⁹ ± 5.8 × 10⁷ vs 4.1 × 10⁹ ± 9.5 × 10⁷ μm³; \( p = 0.044 \)) in the DRLyp/Lyp rats vs control rats (Fig. 5b). Representative images from the OPT of splenic, duodenal and gastric pancreatic lobes from a heterozygote DRLyp/+ rat and a DRLyp/Lyp rat (Fig. 6) present size determination by colour coding. Islets were stained with insulin; red depicts large islets, yellow depicts medium sized islets and white depicts small islets. Additionally, we employed a morphometrical method to assess islet mass in our model [22]. We found no decrease in overall islet mass in the DRLyp/Lyp rats compared with controls (ESM Fig. 1d).

**ATP/ADP ratio is increased in islets from DRLyp/Lyp rats**

GSIS is dependent on mitochondrial metabolism and the resulting
increase in intracellular ratio of ATP/ADP [33]. Therefore, we assessed ATP/ADP ratio in beta cells from DRLyp/Lyp and control rats (Fig. 7a). Interestingly, we observed elevated basal ATP/ADP levels in beta cells from DRLyp/Lyp vs control rats (Fig. 7b; basal Perceval emission at 520 nm: 1333.1 ± 47.3 vs 1094.7 ± 36.4; \( p = 0.0003 \)). Addition of 20 mmol/l glucose raised the ATP/ADP ratio even further in DRLyp/Lyp vs control rats (visualised as \( \Delta_{\text{max}} \) in Fig. 7c; 336.4 ± 31.3 vs 252.2 ± 24.8; \( p = 0.03 \); and slope-increase in Fig. 7d: 4.6 ± 0.5 vs 3.2 ± 0.4; \( p = 0.02 \)). Moreover, AUC for the whole trace was higher in beta cells from DRLyp/Lyp rats (Fig. 7e; \( p = 0.003 \)).

Since mice lacking Glut2 lose the first phase of insulin secretion [34] and display a similar secretory pattern as our model, we investigated Glut2 expression in islets from DRLyp/Lyp and control rats. However, expression of Glut2 was similar in islets from both groups (ESM Fig. 1e).

**Islet morphology and CD3+ cells are similar in DRLyp/Lyp and control rats** To determine changes in islet morphology in DRLyp/Lyp rats, we performed insulin and glucagon staining. Islets in pancreatic sections from both DRLyp/Lyp and control rats displayed normal islet architecture (core of beta cells surrounded by alpha cells; Fig. 8a,c). To confirm previous findings that 40-day-old DRLyp/Lyp rats do not present immune cell infiltration, we performed staining using a CD3+ specific antibody combined with a nuclear DAPI. As expected, staining was sparse, but similar in DRLyp/Lyp and control animals (Fig. 8b,d).

**Discussion**

The present study demonstrates that GSIS is perturbed in the DRLyp/Lyp rat as compared with diabetes-resistant
littermates. The secretory defect was accompanied by significant reductions in the number of medium and small sized islets, and reduced intra-islet blood flow. Notably, these islet-specific derangements were observed at 40 days of age before hyperglycaemia, insulitis and onset of type 1 diabetes.

Type 1 diabetes is associated with the immune-mediated destruction of beta cells, resulting in insulin deficiency. Recent advances have highlighted genetic and functional changes within the beta cell as part of type 1 diabetes pathology [4, 29], suggesting that beta cells may have an inherent sensitivity that possibly makes them susceptible to autoimmune attack. We observed a significant reduction in insulin secretion both in vivo and in vitro in isolated islets from DRLyp/Lyp rats. Indeed, a previous study showed that non-inbred BB rats (BB/Hagedorn; a model where lymphopenia is not present) displayed diminished release of insulin during stimulation with 20 mmol/l glucose in perfused whole pancreas at 50 days of age (before onset of type 1 diabetes) [35]. Similar observations have been made in islets from NOD mice, where insulin secretion immediately after isolation was perturbed (due to insulitis). However, culture of islets from NOD mice over a 5–7 day period improved insulin secretion significantly [31]. Indeed, islets from DRLyp/Lyp rats displayed an improved response to glucose after a culturing period; however, a secretory defect was still evident. Similarly, islets removed from people with new-onset type 1 diabetes show improved GSIS after culture [36]. It is noteworthy, however, that GSIS could not be fully restored in all individuals. A major difference between those studies and ours is that insulitis is not present in 40-day-old DRLyp/Lyp rats. Islets from 40-day-old DRLyp/Lyp rats show reduced expression of the complement inhibitor protein CD59. CD59 is pivotal for normal beta cell exocytosis [37], suggesting that beta cell exocytosis is compromised in DRLyp/Lyp rats. This corresponds to our perfusion data, where islets from DRLyp/Lyp rats display an improved response to 35 mmol/l KCl, suggesting that insulin is not lost, rather that exocytosis is compromised. A previous study highlighted similar findings where non-metabolic secretagogues elicit insulin release in prediabetic conditions and in type 1 diabetes [38]. Additionally, insulin content is not altered in isolated islets from 40-day-old DRLyp/Lyp rats, which further supports this notion.

In prediabetic NOD mice, beta cell dysfunction is suggested to occur as a consequence of early immune cell infiltration and activation of inflammatory cascades [39]. However, the DRLyp/Lyp rats do not display any major infiltration by mononuclear cells until a few days prior to clinical onset of type 1 diabetes [13]. We confirmed this, and islets from DRLyp/Lyp rats did not show increased infiltration of CD3+ cells in pancreatic sections. Moreover, we were unable to detect elevated expression of Il1b, Ifng and Tnf-α in islets from DRLyp/Lyp rats; cytokines that could be indicative of early immune processes within the islets [40, 41].

Beta cell mass is tightly regulated during fetal life, a time point representing a critical window when the appropriate number of beta cells are set in place [42]. A potential weakness in the present study is that we have not investigated neonatal beta cell growth and postnatal expansion of beta cells in our model. It may very well be that DRLyp/Lyp rats are born with a reduced number of beta cells, or fail to expand their beta cell mass during postnatal stages. We observe significant reductions in small and medium sized islets in DRLyp/Lyp compared with control rats, albeit overall islet mass was not changed. A previous study shows that smaller islets contain more insulin per islet volume in situ and secrete insulin more efficiently in vitro [43]. In addition, large islets may be subjected to both hyperplasia and hypoxia [44], resulting in impaired beta cell function. Thus, loss of small and medium sized islets may very well impact insulin secretion. Additionally, OPT has an advantage over more conventional methods, since it can give information on spatial position and volume of individual insulin-expressing islets throughout the pancreas, with high resolution and the opportunity to categorise islets by size [23].

Another important factor influencing beta cell function is nutritional blood status and islet blood flow. This could be considered as the main avenue by which beta cells are kept informed of the body’s nutritional state [45]. We observed reduced intra-islet blood flow in DRLyp/Lyp rats. The importance of this finding for development of type 1 diabetes remains to be determined, but in general lower blood perfusion in islets could compromise beta cell function through hypoxia or limited dispersal of insulin into the systemic circulation [17, 32]. Moreover, decreased blood flow decreases shear stress, which increases the tendency for leucocyte adhesion in venules even in the absence of additional activators [46]. This could promote islet immune cell infiltration. Indeed, a
previous study showed a venular defect in a related rat strain (BB/Wor rat), which supports our findings [18]. Currently, any relationship between blood flow changes and lymphopenia in DRLyp/Lyp rats remains unknown. High basal islet blood flow in diabetes-resistant (and/or wild-type) animals is to a large extent mediated by locally generated nitric oxide from endothelial cells and inhibiting this system decreases blood perfusion [47]. It is noteworthy that studies on islet endothelial cells from young normoglycaemic diabetes-prone and diabetes-resistant BB rats have shown that diabetes-prone rats exhibit considerably lower endothelial cell nitric oxide synthase activity than diabetes-resistant rats [48].

Insulin release is to a large extent dependent on mitochondrial metabolism of glucose and the resulting increase in intracellular ratio of ATP/ADP [33]. Glucose uptake into beta cells is the initial step in GSIS. In rodents this is mediated by GLUT2 [49]. Mice lacking Glut2 lose the first phase of insulin secretion [34]. Thus, both the ATP/ADP ratio and Glut2 expression could influence GSIS in DRLyp/Lyp rats. We observed no changes in Glut2 expression. Intriguingly, however, ATP/ADP levels were elevated in islets from DRLyp/Lyp rats, which could signify a compensatory mechanism as mitochondria are striving to maintain a sufficient ATP/ADP ratio and coupling factors to ensure sufficient insulin release. It may also suggest that the secretory deficiency lies distal of ATP generation (i.e. depolarisation of the plasma membrane/Ca2+ influx or exocytosis). Clearly, more intense research efforts are required in this area.

In summary, our results show that DRLyp/Lyp rats display a secretory defect prior to autoimmune onset of type 1 diabetes. This is manifested by perturbations in insulin secretion in vivo and in vitro, partial loss of beta cell mass and reduced intra-islet blood flow; all of which are factors that influence beta cell function. These changes may be of importance for the development of type 1 diabetes.

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Data availability statement The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Contribution statement The study was designed by MF and ÅL. Blood sampling, glucose analyses and genotyping of BB rats was performed by LÅ, AM and YTS. Islet isolation, data acquisition, analysis and interpretation of perfusion studies and batch incubations were performed by AM, YTS, HB and AB. IVGTTs and analysis thereof was performed by MF and AM. NV performed ATP/ADP measurements/imaging and data analysis. Pancreatic blood flow and intra-islet blood flow experiments and analysis was performed by SU, MQ and POC. Preparation of pancreas for OPT and data analysis was performed by AM, SP and UA. Immunohistochemistry was performed by AM and NW, and analysis thereof was performed by NW. Expression and analysis of genes was performed by AM. The manuscript was drafted by AM and MF. All authors approved the final version of the manuscript. MF is the guarantor of this work.

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