Somatostatin Receptor Type 2 Antagonism Improves Glucagon Counter-regulation In BioBreeding Diabetic Rats

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Abstract

Impaired counterregulation during hypoglycemia in type-1 diabetes (T1D) is in part due to inadequate glucagon secretion. Intra-islet somatostatin suppression of hypoglycemia-stimulated alpha-cell glucagon release plays an important role. We hypothesized that hypoglycemia can be prevented in autoimmune T1D by somatostatin receptor type-2 (SSTR2) antagonism of alpha-cells, which relieve SSTR2 inhibition, thereby increasing glucagon secretion. Diabetic (D) Biobreeding diabetes-prone (BBDP) rats mimic insulin-dependent human autoimmune T1D, whereas non-diabetic (N) BBDP rats mimic prediabetes. D and N rats underwent a 3-h infusion of vehicle vs SSTR2 antagonist (SSTR2a) during insulin-induced hypoglycemia clamped at 3±0.5 mmol/L. D, treated with SSTR2a, needed little or no glucose infusion compared to untreated rats. We attribute this effect to SSTR2a restoration of the attenuated glucagon response. Direct effects of SSTR2a on alpha-cells was assessed by resecting the pancreas, cut into fine slices and subjected to perifusion to monitor glucagon release. SSTR2a treatment enhanced low glucose-stimulated glucagon and corticosterone secretion to normal in D. SSTR2a had similar effects in vivo on N, and promoted glucagon secretion from N and human pancreas slices. We conclude that somatostatin contributes to impaired glucagon responsiveness to hypoglycemia in autoimmune T1D. SSTR2a treatment can fully restore hypoglycemia-stimulated glucagon release sufficient to attain normoglycemia in both diabetic and prediabetic stages.
Introduction

In type-1 diabetes, hypoglycemia is a severe iatrogenic complication caused by intensive insulin treatment required to avoid acute and chronic hyperglycemia and their complications (1). The reason for hypoglycemia is an impairment of counter-regulation, largely but not solely attributed to ‘glucose blindness’ of pancreatic islet alpha-cells, which are unable to secrete glucagon to mobilize glucose from the liver (2). Recent focus on the mechanism for alpha-cell glucose blindness has centered on the role of D-cell somatostatin, culminating in the concept of a defective somatostatin ‘switch-off’ (reviewed in 3). In type-1 diabetes, because of beta-cell destruction, hypoglycemia could no longer induce the decrement reduction in insulin release, a required signal for alpha-cells to release glucagon during hypoglycemia (4,5). Somatostatin from adjacent D-cells is thought to play a minor role in inhibiting alpha-cells in non-diabetic animals and humans because of this dominant inhibitory role of insulin. In the absence of intra-islet insulin in type-1 diabetes, however, somatostatin paracrine inhibition of the alpha-cell becomes manifest (3). In further support for a dominating role for somatostatin’s inhibitory action on D-cell glucagon release, it was very recently shown that tolbutamide inhibition of glucagon secretion was also largely attributable to the somatostatin released from D-cells (6). It would seem that this somatostatin inhibition of glucagon secretion is accentuated in type-1 diabetes. It has been known for some time that exogenously-added somatostatin (SST) could inhibit pancreatic glucagon release (7). This was confirmed in somatostatin receptor type 2 (SSTR2) KO mice showing two-fold greater stimulated glucagon secretion from isolated islets (8). Moreover, the number of D-cells is increased in type-1 diabetic humans and rodents (9). In both species, plasma somatostatin, pancreatic prosomatostatin mRNA and somatostatin protein levels are increased (10,11).
The most convincing evidence has been the effects of somatostatin receptor agonists (12) and antagonists (13) on glucagon secretory response, which identified SSTR-2 as the putative receptor on alpha-cells, as confirmed by studies on SSTR2 KO mice (8). This led to the development of specific SSTR2 antagonists such as PRL-2903 (14), shown to antagonize endogenous SST, resulting in enhancement of arginine-stimulated glucagon secretion from perfused pancreata and perfused islets of non-diabetic rats (13). PRL-2903 is selective for SSTR2 over SSTR3 and SSTR5 by 10- and 20-fold, respectively, and has negligible binding affinity to SSTR1 and SSTR4 (14). We recently explored the therapeutic potential of PRL-2903 on chemically-induced (streptozotocin, STZ) diabetic Sprague-Dawley rats (10). In this model, PRL-2903 normalized not only glucagon, but also corticosterone secretion; the latter is a delayed phase counterregulatory hormone response (10). Most recently, we further demonstrated that SSTR2 antagonism can ameliorate or prevent hypoglycemia in STZ diabetic rats exposed to antecedent hypoglycemia (15).

The STZ-treated rat model, however, does not genuinely mimic human type-1 diabetes, which is an autoimmune disorder. Furthermore, this model does not require insulin treatment, while human type-1 diabetes does. In this study, we have employed the insulin-requiring Bio-breeding diabetic prone (BBDP) rat as an authentic autoimmune model of human type-1 diabetes (16,17) to test the therapeutic actions of SSTR2 blocker PRL-2903. BBDP rats are prediabetic prior to developing diabetes (18,19) and their islet morphology shows infiltration with inflammatory cells (insulinitis) before the disappearance of beta-cells (18). This was sufficient to cause reduced insulin secretion from isolated islets, accounting for the glucose intolerance when challenged with hyperglycemic clamps (19). We concluded that in the overt diabetic state with beta-cell destruction and the prediabetic state with insulinitis (used in this study as Control), BB rat alpha-
cell somatostatin receptor signaling could be perturbed, but corrected by the SSTR2 antagonist. Indeed, using hyperinsulinemic hypoglycemic clamp technique, PRL-2903 restored glucagon secretion in the diabetic BBDP rats. Central (hypothalamic) and peripheral counterregulatory pathways (catecholamines, corticosterone) are also influenced by SST, and themselves affect glucagon secretion (10,20), thus could confound the interpretation of the direct actions of PRL-2903 on alpha-cells. Since islets could not be reliably isolated from type-1 diabetes models, we deployed the pancreas slice technique (21,22), and used perifusion to directly measure islet hormone secretion. We showed that PRL-2903 restored glucagon secretion from BBDP rat pancreas slices and normal human pancreas slices and isolated islets.

**Research Design and Methods**

**Animals.** Male diabetes prone BB/Wor rats (Biomere, Worcester, MA, USA) were housed in a sterile animal facility, fed standard diet, and maintained on a 12-h/12-h day/night cycle. Age-matched BBDP rats which did not become diabetic at age range of 60-150 days were used as controls. BBDP rats that became diabetic (random BS$> 22$mmol/L) were treated by subcutaneous implants of 1.5-2 insulin pellets (LinShin Canada, Toronto, ON, Canada). All procedures were in accordance with Canadian Council on Animal Care Standards and approved by the University of Toronto Animal Care Committee.

**Human pancreas specimens.** Normal portions of human pancreas resected from patients undergoing surgical resection for pancreatic cancer were obtained from the Toronto General Hospital, with pre-operative written consent obtained from source patients. The surgical pathology laboratory, after assessing the resected tissue, immediately places a normal portion in extracellular solution; our laboratory is then called to retrieve the specimen to process.
Experiments are performed within hours. Normal human pancreatic islets were kindly provided by Patrick MacDonald (Alberta Diabetes Institute, University of Alberta, Edmonton, Canada). All procedures involving human pancreas tissues were approved by the Research Ethics Boards of the University Health Network and University of Toronto.

**Somatostatin receptor type-2 antagonist (SSTR2a).** The SSTR2a peptide, PRL-2903, was synthesized by David Coy (a co-author), and demonstrated to be selective for SSTR2 (14), and this was supported by our recent studies using this compound (10,15). The SSTR2a was dissolved in 1% acetic acid and diluted to the calculated dose with 0.9% saline.

**Hypoglycemic clamp experiments.** The BBDP rats were studied in 4 groups: diabetic vehicle (n=8), diabetic+SSTR2a (n=8), non-diabetic vehicle (n=5) and non-diabetic+SSTR2a (n=6). The surgery and clamp procedures were performed as described in our previous study in STZ-treated rats (10). Surgery was performed 7 days prior to the clamp experiments; for diabetic rats, 14 days after insulin pellet implantation. Rats were fasted overnight prior to the clamp study, then weighed. Under general anaesthesia by isoflurane inhalation, the left carotid artery and right jugular vein were catheterized for blood sampling and infusion of test substances, respectively; and then acclimatized for 1 hour with great care to avoid any stress throughout the clamp procedure. After obtaining baseline blood samples at t = -60 min, the rats underwent a 3-hour infusion of vehicle (1% acetic acid in 0.9% saline) or SSTR2a (1500 nmol/kg/h) at an infusion rate of 1ml/h with a digital syringe infusion pump (Harvard Apparatus, Holliston, MA). In order to induce hypoglycemia to a target level of 3±0.5mmol/L, insulin infusion at the constant rate of 20-50 mU/kg/min together with variable rates of glucose infusion were started at t = 0 min. During the clamp, blood samples were obtained from the carotid catheter every 10 minutes, one set into capillary tubes coated with Kalium-EDTA (Microvette CB 3000, Sarstedt...
Inc., Montreal, QC, Canada), immediately centrifuged (room temperature), then plasma glucose levels determined by glucose analyzer (Analox Glucose analyzer, Analox instrument, London, UK); and another set for hormone measurements.

Right after the clamp study, the rats were sacrificed and whole pancreata dissected, frozen on dry ice and transferred to -80°C for latter determinations of somatostatin and glucagon total pancreatic content measurements. Whole pancreata of age-matched BBDP diabetic ± insulin pellet implantation (n=6 for each group) and non-diabetic rats (n=5) were also dissected and preserved without undergoing hypoglycemic clamps, awaiting total content hormone measurements.

**Plasma hormone measurements.** Blood samples were obtained at t= -60 min, then every 30 minutes from t=0 min in ice-chilled tubes containing 5µl of 100mM EDTA (Ethylenediaminetetraacetic acid) solution and 30 KIU aprotinin (APR600, BioShop Canada Inc., Burlington, ON, Canada); followed by centrifugation at 12000 rpm at 4°C to separate the plasma. The plasma was aliquoted and immediately frozen on dry ice and transferred to -80°C awaiting hormone measurements for glucagon (radioimmunoassay kit, EMD Millipore, Darmstadt, Germany), corticosterone (ELISA kit, ALPCO Diagnostics, Salem, NH), nor/epinephrine (2-Cat Plasma ELISA kit, Labor Diagnostica Nord GmbH & Co. KG, Nordhorn, Germany), and somatostatin (extraction free, rat somatostatin-14 enzyme immunoassay kit, Bachem Group, Bubendorf, Switzerland).

**Total pancreatic glucagon and somatostatin protein content.** The frozen pancreata were placed in acid-ethanol mixture (1.5%HCl in 70% EtOH), incubated twice overnight at -20°C, homogenized, then centrifuged, and neutralized by 1M pH 7.5 Tris buffer. Somatostatin and
glucagon levels were determined as above, and normalized to total pancreatic protein content
determined by modified Lowry method.

**Pancreatic slices preparation and perifusion.** Pancreas slices were prepared as recently
described (21,22). Upon sacrifice, the rat abdominal cavity was opened, the common bile duct
was then identified, clamped and injected with 3 ml of 37°C low-melting 1.9% agarose gel
(15517-022, Invitrogen, Camarillo, CA). The pancreas was resected, cut into smaller pieces, then
embedded in 1.9% agarose gel and cooled down to become solidified. The tissue blocks were
placed in Carbogen (5% CO₂, 95% O₂) bubbled ice-cold extracellular solution, then sliced into
4×4 mm, 140µm-thick slices with a vibrating blade microtome (Vibratome, Leica Microsystems,
Mannheim, Germany) at a blade frequency of 70 Hz. The extracellular solution bathing the
pancreas slices was composed of (in mmol/L) 125 NaCl, 2.5 KCl, 1 MgCl₂, 2 CaCl₂, 1.25
NaH₂PO₄, 26 NaHCO₃, 2 Na pyruvate, 0.25 ascorbic acid, 3 myo-inositol, 6 lactic acid, 7
glucose. Human pancreas slices were similarly prepared after embedding into the agarose gel.

10 slices of the BB rat pancreas and 15-20 slices of human pancreas were loaded into the
perifusion chambers and perifused with a HEPES-balanced KRBB solution (KRBH) containing
in mmol/L: 135 NaCl, 3.6 KCl, 0.50 NaH₂PO₄, 0.5 MgCl₂, 1.5 CaCl₂, 2 NaHCO₃, 10 HEPES,
and 1 g/L BSA, at a flow rate of 1 ml/min, and samples collected at 1-min intervals. As
indicated in the study protocols, the perifusion solution would contain 1 or 7mmol/L glucose,
with or without SSTR2a (PRL-2903, 30 µmol/L) or arginine (20 mmol/L). Each study was
preceded with a 20-minute equilibration period at 7mmol/L glucose. The BB rat pancreas slice
study was carried out in 4 groups: diabetic, diabetic+SSTR2a, non-diabetic, non-
diabetic+SSTR2a. Samples were collected at 1-min intervals into ice chilled tubes containing
1000 KIU aprotinin, then frozen and stored at -80°C awaiting determination of rat glucagon and
human insulin levels (RIA kits, EMD Millipore). At the end of the experiments, the slices were collected from each chamber and preserved in acid-ethanol mixture (1.5% HCl in 70% ethanol) and kept at -80°C for later determination of total glucagon and insulin content. The perifusion setup enables dilution of acinar enzymes that would otherwise degrade the secreted hormones. This is not the case in batched static incubation studies of pancreatic slices.

**Immunohistochemistry.** Identification of SSTR2 in the islets within the pancreas tissue was with monoclonal anti-SSTR2 rabbit antibody (1:200, Cat# 3582-1, Epitomics, Burlingame, CA) by immunohistochemical analysis as described by Taniyama et al (23), and by immunofluorescence double labeling with mouse monoclonal anti-glucagon (GeneTex Inc., San Antonio, TX) and anti-insulin antibodies (Sigma-Aldrich, St. Louis, MO) as we had described (21,22). The latter was viewed with a Leica DMIRE2 inverted fluorescence microscope system (Mannheim, Germany).

**Statistical analysis.** Data are presented as means ± SEM for a given number of observations. Groups of data were compared using either using a two-tailed unpaired Student’s t-test or with ANOVA followed by a Tukey’s HSD post-hoc test (Graphpad, PRISM software, San Diego, CA) adjusting for multiple comparison where necessary. Significance was assigned when $P$ values were <0.05.

**Results**

**Hypoglycemic clamp experiments.**

**Glucose and insulin requirements.** As mentioned in the methods section, the controls used in this study are non-diabetic BBDP rats, which because of islet inflammation (insulinitis) may be considered as in a prediabetic state, exhibiting mild glucose intolerance when challenged with
experimental hyperglycemia (19). This could explain some of the responses to hypoglycemia observed in our study. At the start of the experiment (Fig. 1A, t=−60min) (i.e. before insulin, glucose or SSTR2a infusion), blood glucose levels were not significantly different between the 4 groups (non-diabetic vehicle: 7.84±0.27 mmol/L, non-diabetic SSTR2a: 8.05±0.51 mmol/L, diabetic vehicle: 7.19±0.43 mmol/L, diabetic SSTR2a: 7.43±0.43 mmol/L). At t=0, the non-diabetic SSTR2a (7.55±0.30 mmol/L) group was also not significantly different from non-diabetic vehicle group (6.97±0.25 mmol/L). This was also true for the diabetic rats (vehicle: 6.41±0.32; SSTR2a: 6.92±0.58 mmol/L). The diabetic rats had generally lower glucose levels since they were treated with insulin pellets.

All four groups were then treated with insulin and variable glucose infusion to reach the target hypoglycemic blood level of 3±0.5 mmol/L within 70 minutes after the start of the insulin infusion and maintained at this level by the variable glucose infusion until the end of the experiment (Fig. 1A). To maintain this glucose level, the diabetic vehicle group required very high glucose infusion (AUC: 1157±146.6), as would be expected based on their poor counterregulation to hypoglycemia (Fig. 1B). In contrast, all other groups required very low or no glucose infusion (AUCs all <33). The insulin infusion rates were not significantly different between the diabetic vehicle (25±1.89 mU/kg/min), diabetic SSTR2a (25.56±1.75 mU/kg/min) and non-diabetic vehicle (26±2.4 4 mU/kg/min) groups; however, the insulin requirement for non-diabetic SSTR2a group, was higher at 50 mU/Kg/min to maintain the target hypoglycemic level. This could reflect the fact that non-diabetic BB rats were insulin-resistant (19), which was accentuated by SSTR (see Discussion).

**Effects on hormone secretion: glucagon, corticosterone, catecholamine release and somatostatin.** In the diabetic vehicle group, glucagon secretion during hypoglycemia was low
(AUC: 8,982±715, Fig. 2A) compared to the non-diabetic vehicle group (AUC: 20,679±2201). With SSTR2a treatment, the diabetic SSTR2a group showed an increase in glucagon secretion to (AUC: 21,722±2962), which was 2.4 fold that of diabetic vehicle group, indicating full restoration in glucagon secretion. SSTR2a treatment also enhanced the glucagon secretion in non-diabetic SSTR2a group (AUC: 69,897±4806) to ~3.3 fold compared to non-diabetic vehicle.

For corticosterone secretion (Fig. 2B), diabetic vehicle (AUC: 16,025±924.3) was not significantly different from non-diabetic vehicle (12,062±507.6). When treated with SSTR2a, the-diabetic SSTR2a group showed increase in corticosterone secretion (22,341±2195), but SSTR2a did not affect corticosterone secretion in non-diabetic SSTR2a group (13,255±1186). Epinephrine and norepinephrine responses to hypoglycemia (Fig. 2C) were not significantly different in the 4 groups.

Plasma somatostatin levels (Fig. 2D) were not different between the diabetic vehicle (AUC: 78.85±18.68) and diabetic SSTR2a (89.52±24.64) groups, and neither were significantly different from non-diabetic vehicle (42.32±9.39) group. Interestingly, SSTR2a was able to increase somatostatin secretion from the non-diabetic SSTR2a group (AUC: 290±26.29) by 6.8 fold compared to non-diabetic vehicle, which was not seen in the diabetic groups, suggesting that somatostatin secretion might be perturbed in the diabetic rats or more responsive in the non-diabetic BBDP rats.

**Pancreatic glucagon and somatostatin protein content.** We assessed the pancreatic glucagon and somatostatin content in non-diabetic and diabetic BBDP rats at baseline (without or with treatment with insulin pellets to prevent hypoglycemia) (Fig. 3A); and in those that were subjected to the hypoglycemic clamps (Fig. 3B).
No hypoglycemic clamp. Pancreatic glucagon (Fig. 3A left panel) and somatostatin (right panel) protein content in insulin pellet-treated diabetic BBDP rats were both lower than untreated diabetic and non-diabetic BBDP rats. This would suggest that insulin treatment to induce more normoglycemic control would reduce the demand on alpha-cells and D-cells to synthesize more glucagon and somatostatin, respectively. This could reflect the absence of endogenous insulin.

Following hypoglycemic clamp. Pancreases resected from non-diabetic and insulin pellet-treated diabetic BBDP rats after the insulin-induced hypoglycemic clamp (Fig. 3B left panel) showed that whole pancreatic glucagon protein content was lower in the diabetic SSTR2a group (11.94±0.67 ng/mg protein) compared to the diabetic vehicle group (18.28±1.36 ng/mg protein, p<0.001), and tended to be lower compared to the non-diabetic groups, although these differences failed to reach statistical significance after adjusting for multiple comparisons. The reduction in glucagon content between the SSTR2-treated and untreated diabetic rats was likely resultant from the SSTR2a promoting the release of glucagon from the animals during hypoglycemia.

SSTR2a treatment did not affect the pancreatic somatostatin content (Fig. 3B right panel) in the diabetic groups (vehicle: 64.65± 5.21; SSTR2a: 52.11± 2.89 pg/mg protein). However, the somatostatin content in the non-diabetic group (81.95±8.86 pg/mg protein) was increased by SSTR2a treatment (non-diabetic SSTR2a: 118.7±13.27 pg/mg protein) (Fig. 2D). At the present time, we cannot clarify why pancreatic somatostatin content immediately after the clamp was elevated in the non-diabetic SSTR2a group as compared to the other three groups.

BBDP rat pancreatic slice perifusion. We first assessed the SSTR2 distribution in the islets of BBDP rat pancreatic sections (Fig. 4A). In non-diabetic BBDP rat islets, SSTR2-containing alpha-cells were distributed more towards the islet periphery. In the shrunken diabetic BBDP
islets resulting from reduced beta-cell mass, SSTR2-containing alpha-cells were redistributed more to the islet core. SSTR2 was present only in glucagon-staining alpha-cells and not in insulin-staining beta-cells (few beta-cells remaining in diabetic BB rat islet). In Fig. 4B, after initial equilibration, low glucose (1 mmol/L) stimulated diabetic BBDP rat pancreas slices to release only a small amount of glucagon secretion (Secretion rate: 1.29±0.12), and this was inhibited by 7 mmol/L glucose (0.92±0.26). SSTR2a treatment increased 1 mmol/L glucose-stimulated glucagon secretion rate (3.16±0.40) by 2.4 fold. Arginine (at 7 mmol/L glucose) plus SSTR2 given together with the antagonist greatly increased glucagon secretion rate. These in vitro results indicate that the increased blood levels of glucagon observed in Fig. 2A in both diabetic and non-diabetic BBDP rats were largely attributed to the direct effects of SSTR2a on the pancreatic alpha-cells.

**Human pancreatic slices and islet perifusion.** We next assessed whether our finding in BBDP rats applied to human pancreatic alpha-cells. We were not able to obtain pancreas samples from type-1 diabetic patients, and thus could only assess normal pancreas, obtained from pancreatic surgical resections of pancreatic cancer patients, on which we performed immunohistochemistry (Fig. 5A) and pancreas slice perfusion assay (Fig. 5B) to simulate the results obtained from the BBDP rats. We also employed the conventional human isolated islets, and subjected to islet perifusion assay (Fig. 5C). A recent report demonstrated that SSTR2 are in both human alpha- and beta-cells (24). We thus first assessed the distribution of SSTR2 in the human pancreas section, and indeed found SSTR2 to be in both glucagon-staining alpha-cells and insulin-staining beta-cells (Fig. 5A), and both cell types are mixed within the islet including the islet core. Thus, we examined whether SSTR2a could influence both glucagon and insulin secretion.
Shown are two experiments for human pancreas slices (Fig. 5B). 1 mmol/L glucose-stimulated glucagon secretion was enhanced by the addition of SSTR2a in both experiments (top). It was difficult to obtain pancreas specimens from pancreatic cancer resections, hence we also employed the use of conventional human islets (Fig. 5C). We observed similar results of SSTR2 potentiation of glucagon secretion (top, AUCs, 1 mmol/L glucose+SSTR2a: 9.53±0.77 vs 1 mmol/L glucose: 4.10±1.11, N=4) to 2.3 fold. Low glucose did not stimulate insulin release from human pancreas slices (bottom, Fig. 5B) nor isolated islets (not shown). Addition of SSTR2a had no significant effect (isolated islets AUCs, 1 mmol/L glucose+SSTR2a: 11.65±2.21 vs 1 mmol/L glucose: 13.19±1.79, N=4, not significant). This is likely because 1mmol/L glucose was non-stimulatory on beta-cells and stimulatory glucose concentrations are required for SSTR2a to potentiate insulin release (12). Arginine (Fig. 5B) evoked release of more glucagon (at stimulatory glucose) and insulin (at inhibitory glucose), which serve as positive control and an indicator that the slices were healthy.

Discussion

Previously, in STZ-induced diabetic rats, we have shown that antagonising SSTR2 fully restores the glucagon and corticosterone counterregulation to insulin-induced hypoglycemia (10). The STZ model is not a genuine disease model for humans in which type-1 diabetes is an autoimmune disorder that requires continuous insulin treatment. In contrast, in STZ rats, hyperglycemia is induced chemically and in our previous experiments they were not insulin-treated (10). Nonetheless, we assume that the hyperinsulinemic hypoglycemic clamp in this model closely mimics iatrogenic hypoglycemia in type-1 diabetes (2). In the present study, we demonstrate for the first time that antagonising the SSTR2 bound to alpha-cells in insulin-treated
autoimmune type-1 diabetic BBDP rats (16-19), fully restores the glucagon counterregulation to insulin-induced hypoglycemia. This restoration in glucagon and corticosterone secretion in vivo has major impact on improving glucose counteregulation in diabetic rats, as SSTR2a-treated animals did not require glucose infusion during the hypoglycemic clamp while the untreated diabetic BBDP rats required large amounts of glucose infusion.

Our controls are non-diabetic BBDP rats, which have a low-grade inflammation in the islets prior to destruction of beta-cells, causing rapid progression to severe diabetes requiring exogenous insulin to survive (18,19), thus mimicking the developing pathology in human type-1 diabetes. We therefore decided that this prediabetic state of the BBDP rat was a more appropriate and clinically-relevant control for diabetic rats than normal Sprague Dawley rats that do not have inflammation in the islets. Interestingly, we also observed that SSTR2a treatment of the non-diabetic BBDP rats enhances glucagon secretion during insulin-induced hypoglycemia. However, the previous study on normal Sprague Dawley (10) did not show any enhancement of glucagon secretion by SSTR2a treatment. This could reflect the fact that non-diabetic BBDP rats are actually in a prediabetic stage of the disease. In fact, when non-diabetic BBDP rats were challenged with experimental hypoglycemia, they showed glucose intolerance that was attributed to reduced insulin release from the islets (19). We hypothesize, therefore, that in addition to the effects of exogenous insulin, inflammatory mediators released during prediabetic insulinitis (18) may have sensitized alpha-cells to somatostatin inhibition.

We also measured epinephrine, norepinephrine and corticosterone responses to hypoglycemia after SSTR2a treatment. We did not observe any change in epinephrine and norepinephrine responses by SSTR2a treatment. We did, however, observe enhanced corticosterone response to hypoglycemia. This was similarly observed in our previous study with STZ-treated Sprague
Dawley rats (10). Improvement of cortisol responses is important during prolonged hypoglycemia, especially during sleep in children with type 1 diabetes.

Pancreatic glucagon is not decreased following hypoglycemia in the diabetic BBDP rats. Thus, the defect in glucagon counterregulation may not be due to a decrement in glucagon synthesis but rather a deficiency in glucagon release, which has been shown in STZ diabetic rats (10). SSTR2a administration decreased the pancreatic glucagon protein content, while the secretory plasma glucagon levels in response to hypoglycemia were restored. This finding suggests that SSTR2a may increase alpha-cells potency to release glucagon in response to hypoglycemia. Indeed, we postulate that the main reason for the high exogenous insulin requirement for the non-diabetic SSTR2a group is the enhanced plasma glucagon levels as a result of SSTR2 blockade, perhaps due to inflammation of the islets.

Pancreatic somatostatin protein content in the untreated diabetic rats remained unchanged compared to the non-diabetic rats and insulin treatment further decreased the pancreatic somatostatin content in diabetic rats. The fact that the insulin-treated diabetic BBDP rats do not have elevated pancreatic somatostatin and yet SSTR2a can normalize hypoglycemia, demonstrates efficiency of the antagonist even when pancreatic and plasma somatostatin are normal. From our data in slices of human pancreas, it appears that the effect of SSTR2a is magnified because it is known that in human diabetics, pancreatic and plasma somatostatin are increased (11). It is known that the main source of plasma-somatostatin originates from the gastrointestinal tract (23,24), which could add to increased plasma somatostatin during hypoglycemia.

In order to isolate the effects of hypoglycemia and the antagonist on glucagon release, we used for the first time, fresh, thin, pancreatic slices (19, 20) in the perifusion setting. We were
able to address the real-time glucagon secretory responses to hypoglycemia in both prediabetic (non-diabetic BB rat) and diabetic conditions, devoid of other intervening factors present in vivo such as effects of the central and autonomic nervous systems and circulating amino acids, which can also stimulate glucagon release. Similar differences in glucagon secretion between in vivo experiments and whole pancreatic perfusions have been observed previously (25,26). The advantage of slices is that the pancreatic islets after type-1 diabetes autoimmune injury and scarring are considerably smaller in size, and have a much distorted shape and architecture. Any attempt to isolate them through exposure to enzymatic digestion will damage them (22).

Furthermore, in contrast to human islets, alpha-cells in rodents are located mostly in the islet periphery (27), which would be even more prone to injury from the isolation procedures. Using pancreatic slices perifusion, we observed that SSTR2a significantly enhances the glucagon response during hypoglycemia both in diabetic and non-diabetic BBDP rats. This finding further confirms the paracrine inhibitory effect of pancreatic somatostatin on glucagon secretion, which is mediated via the SSTR2 on alpha-cells.

We also observed that the basal glucagon secretion (at 7mM glucose) is moderately enhanced in both diabetic and non-diabetic BBDP rats. A recent model was postulated to explain the basal hyperglucagonemia in type-1 diabetes, which is that glucagon secretion has two components (28). The first is under auto-feedback control which drives pulsatile glucagon counterregulation; the second is independent of this feedback on basal glucagon secretion whereby its increase can suppress the glucagon counterregulation. When considering SSTR2a as a potential therapeutic for preventing hypoglycemia in type-1 diabetes, it is very important to find out whether SSTR increases basal and/or postpandial glucagon secretion, the latter of which may be driven by a rise in amino acid levels. Our previous study on STZ diabetic rats indicated that SSTR2a infused for
4 hours during basal conditions in vivo resulted only in a small, transient glucagon increase, without affecting corticosterone and catecholamine levels in plasma (10). Most importantly, glucose concentration remained unchanged.

It was previously demonstrated that SSTR2a treatment reverses the inhibitory effects of SSTR2 agonism on insulin and glucagon secretion in isolated human islets (12). However, the effectiveness of SSTR2a on enhancing glucagon secretion during hypoglycemia in human pancreas was unknown. Employing thin pancreatic slice perifusion technique, for the first time, we demonstrate that SSTR2a enhances the glucagon secretory response to hypoglycemia. We observed similar results with isolated human islet perifusion, which also serves to validate the human pancreas slice perifusion assay. This model is ideal for future studies on pancreas specimens from type-1 diabetic patients. Simultaneous measurements of insulin secretion from the human pancreatic slices and isolated islets during hypoglycemia showed no effect by SSTR2 blockade, since stimulatory glucose concentrations are required for SSTR2a potentiated insulin release (12). It was shown that SSTR2 is the functionally-predominant somatostatin receptor on both alpha- and beta-cells in human pancreas (12, 24). Our findings, taken together with those studies, suggest that SSTR2a can improve glucagon secretion in type-1 diabetes where only alpha-cells are present, and that it may also improve insulin secretion when there are still some residual beta-cells.

The potent effects of the SSTR2a on normal human pancreas may be due to the fact that the population of D-cells in human islets are about twice that of rodent islets, whereas beta-cells populations are less in human islets than in rodent islets (27). This would suggest that the proportionate inhibitory effects of insulin and somatostatin on human alpha-cells may be different from that on rodent alpha-cells, with somatostatin probably having a greater effect on
the former. Since SSTR2a can restore glucagon secretion in BBDP rats, preventing insulin-induced hypoglycemia, there could be important clinical implications in employing SSTR2 antagonists to prevent iatrogenic hypoglycemia in type-1 diabetic patients who are under tight glycemic control (1).

**Authors’ contribution:** N.K. and T.Q. performed most of the experiments, their design and data analysis. T.L., M.O., Y.H., and T.T. contributed some experiments and to discussion. D.H.C, M.R. and M.S.C. contributed reagents and to the discussion. N.K., M.V. and H.Y.G. wrote the manuscript.

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Dr. Herbert Y. Gaisano is the guarantor of this work and, as such, has full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. The authors have no conflict of interest.

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Figure legends:

**Figure 1.** (A) Plasma glucose levels during hyperinsulinemic hypoglycemic clamp experiments (Vehicle or 1500 nmol/kg/hr SSTR2a and 20-50 mU/kg/min insulin). (B) Glucose infusion rates during hyperinsulinemic hypoglycemic clamp experiments. Black squares: Diabetic SSTR2a, n=8; White squares: Diabetic vehicle, n=8; Black circles: Non-diabetic SSTR2a, n=6; White circles: Non-diabetic vehicle, n=5. Data are presented as means ± SEM.

**Figure 2.** Plasma hormone levels during hyperinsulinemic hypoglycemic clamp experiments (Vehicle or 1500 nmol/kg/hr SSTR2a and 20-50 mU/kg/min insulin) and corresponding AUCs. (A) Effect of SSTR2a on glucagon response. (B) Effect of SSTR2a on corticosterone response. (C) Effect of SSTR2a on epinephrine response. (D) Plasma somatostatin levels. Black squares: Diabetic SSTR2a, n=8; White squares: Diabetic vehicle, n=8; Black circles: Non-diabetic SSTR2a, n=6; White circles: Non-diabetic vehicle, n=5. AUCs were calculated using Prism software (GraphPad software, San Diego, CA). Black bars: Diabetic SSTR2a, n=8; White bars: Diabetic vehicle, n=8; Vertically striped bars: Non-diabetic SSTR2a, n=6; Horizontally striped bars: Non-diabetic vehicle, n=5. Data are presented as means ± SEM. (**: p<0.01 and *: p<0.05)

**Figure 3.** Whole pancreatic protein contents of glucagon and somatostatin. (A) Non-diabetic and Diabetic BBDP rats (with and without insulin pellet implants) that have not undergone clamp experiments. Black bars: Insulin-treated Diabetic BBDP rats, n=6; White bars: Untreated Diabetic BBDP rats, n=6; Horizontally striped bars: Non-diabetic BBDP rats, n=5. Pancreases were obtained immediately following the hyperinsulinemic hypoglycemic clamp experiments. Diabetic BBDP rats had been treated with insulin pellet implants before the clamp experiments. Black bars: Diabetic SSTR2a, n=8; White bars: Diabetic vehicle, n=8; Vertically striped bars: Non-diabetic SSTR2a, n=6; Horizontally striped bars: Non-diabetic vehicle, n=5. Data are presented as means ± SEM. (**: p<0.01 and *: p<0.05)

**Figure 4.** Effects of SSTR2a on glucagon secretion from pancreatic slices of BBDP Diabetic and Non-diabetic rats. Immuno- (i, brown is SSTR2 labeling) and immunofluorescence (red – SSTR2; green – glucagon (ii) or insulin (iii); merged images) histochemistry of the localization of SSTR2 to islet alpha-cells in BBDP Diabetic (A) and Non-diabetic (B) rats pancreas slices. Insets in (ii) are enlarged views showing SSTR2 and glucagon colocalization. In Aiii, the few surviving beta-cells in Diabetic BBDP rats do not contain SSTR2, as is also true in Non-diabetic rats (data not shown). Scale bar, 100µm. Glucagon secretion from pancreatic slices of (C) Diabetic BBDP rats, n=6, and (D) Non-diabetic BBDP rats, n=6. Black circles and bars: with SSTR2a treatment, white circles and bars: no SSTR2a treatment. Relative glucagon secretion was calculated as the glucagon secretion from the slices normalised to the total glucagon content of the slices. Secretion rates were calculated using Prism software (GraphPad software, San Diego, CA). Data are presented as means ± SEM. (**: p<0.01 and *: p<0.05) (G: Glucose, A: Arginine)

**Figure 5.** Effects of SSTR2a on glucagon and insulin secretion from normal human pancreatic slices and isolated islets. (A) Immuno- (i, brown is SSTR2 labeling) and immunofluorescence (red – SSTR2; green – glucagon (ii) or insulin (iii); merged images) histochemistry of the localization of SSTR2 to islet alpha-cells (ii) and beta-cells (iii) in normal human pancreas slices. Inset in (ii) is an enlarged view showing SSTR2 and glucagon colocalization. Scale bar, 100µm. (B) Relative glucagon (top) and insulin (bottom) secretion from pancreatic slices. The black and
white circles represent two sets of perifusion. (C) Relative glucagon secretion from isolated pancreatic islets, n=4. Black bars: AUC of 1mM G +SSTR2a condition, White bars: 1 mM glucose condition. Relative glucagon or insulin secretion was calculated as the glucagon/insulin secretion from the slices/islets normalised to the total glucagon/insulin content of the slices/islets. AUCs were calculated using Prism software (GraphPad software, San Diego, CA). Data are presented as means ± SEM. (**: p<0.01 and *: p<0.05) (G: Glucose, A: Arginine)
Plasma glucose (mmol/l)

Target hypoglycemia: 3 ± 0.5 mmol/l
**A**

IV Vehicle or 1500 nmol/kg/hr SSTR2a

IV insulin or variable glucose

**B**

Diabetic +SSTR2a

Diabetic vehicle

Non-diabetic + SSTR2a

Non-diabetic vehicle

**C**

Diabetic +SSTR2a

Non-diabetic + SSTR2a

Non-diabetic vehicle

**D**

Diabetic +SSTR2a

Non-diabetic + SSTR2a

Non-diabetic vehicle

Diabetes
Pancreatic glucagon (ng/mg protein) vs Ins. pellet and Diabetes

A. No hypoglycemic clamp

- Ins. pellet: +, -, -
- Diabetes: +, +, -

B. Following hypoglycemic clamp

- SSTR2a: +, -, +, -
- Diabetes: +, +, -

Pancreatic somatostatin (pg/mg protein)
C. Diabetic BBDP rat pancreatic slices

Relative glucagon secretion from the slices

\[ \text{Time (min)} \]

\[ \text{Relative glucagon secretion rates from the slices (pg/ml/min)} \]

D. Non-diabetic BBDP rat pancreatic slices

Relative glucagon secretion from the slices

\[ \text{Time (min)} \]

\[ \text{Relative glucagon secretion rates from the slices (pg/ml/min)} \]
For Peer Review Only

**Human pancreatic slices**

- **Relative glucagon secretion** from the slices
  - AUC
  - Set 1: **Set 2:**

- **Relative Insulin secretion** from the slices
  - AUC

**Human pancreatic islets**

- **Relative glucagon secretion** from the islets
  - AUC

Diabetes