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Effects of somatostatin receptor type 2 antagonism during insulin-induced hypoglycaemia in male rats with prediabetes

Emily G. Hoffman BSc[1] | Ninoschka C. D'Souza MSc[1] | Julian Aiken PhD[1] | Sara Atherley BSc[1] | Richard Liggins PhD[2] | Michael C. Riddell PhD[1]

[1]School of Kinesiology and Health Science, Muscle Health Research Centre, York University, Toronto, Ontario, Canada

[2]Zucara Therapeutics, Vancouver, British Columbia, Canada

Correspondence
Emily G. Hoffman, School of Kinesiology and Health Science, Muscle Health Research Centre, York University, 4700 Keele Street, Toronto, ON M3J 1P3, Canada.
Email: hoffman1@my.yorku.ca

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Abstract

Aims: To examine if glucagon counterregulatory defects exist in a rat model of prediabetes (pre-T2D) and to assess if a selective somatostatin receptor 2 antagonist (SSTR2a), ZT-01, enhances the glucagon response to insulin-induced hypoglycaemia.

Materials and methods: Hyperglycaemia was induced in 8- to 9-week-old male, Sprague-Dawley rats via 7 weeks of high-fat diet followed by a single, low-dose intraperitoneal injection of streptozotocin (30 mg/kg). After 2 weeks of basal insulin therapy (0-4 U/d insulin glargine, administered subcutaneously [SC]) to facilitate partial glycaemic recovery and a pre-T2D phenotype, n = 17 pre-T2D and n = 10 normal chow-fed control rats underwent the first of two hypoglycaemic treatment-crossover experiments, separated by a 1-week washout period. On each experimental day, SSTR2a (3 mg/kg ZT-01, SC) or vehicle was administered 1 hour prior to insulin-induced hypoglycaemia (insulin aspart, 6 U/kg, SC).

Results: Glucagon counterregulation was marginally reduced with the induction of pre-T2D. Treatment with SSTR2a raised peak plasma glucagon levels and glucagon area under the curve before and after insulin overdose in both and pre-T2D rats. Blood glucose concentration was elevated by 30 minutes after SSTR2a treatment in pre-T2D rats, and hypoglycaemia onset (≤3.9 mmol/L) was delayed by 15 ± 12 minutes compared with vehicle ($P < 0.001$), despite similar glucose nadirs in the two treatment groups (1.4 ± 0.3 mmol/L). SSTR2a treatment had no effect on blood glucose levels in the control group or on the hypoglycaemia-induced decline in plasma C-peptide levels in either group.

Conclusions: Treatment with an SSTR2a increases glucagon responsiveness and delays the onset of insulin-induced hypoglycaemia in this rat model of pre-T2D where only a modest deficiency in glucagon counterregulation exists.

KEYWORDS
glucagon, glucose counterregulation, hypoglycaemia, prediabetes, somatostatin, somatostatin receptor 2 antagonist, type 2 diabetes

* Reprint Request: Michael C. Riddell, PhD, School of Kinesiology and Health Science, Muscle Health Research Centre, York University, 4700 Keele Street, Toronto, ON M3J 1P3, Canada.

1 | INTRODUCTION

Glucagon, secreted by pancreatic α-cells, prevents and/or attenuates the development of hypoglycaemia by mobilizing glucose from the liver.[1] In type 1 diabetes (T1D), the counterregulatory glucagon response is typically absent within a few years of diagnosis, predisposing affected individuals to insulin-induced hypoglycaemia.[2] While the natural history of this acquired α-cell defect is not well characterized in type 2 diabetes (T2D), owing, at least in part, to heterogeneity in disease phenotype and progression, it is well established that hypoglycaemia, secondary to the intensive use of insulin analogues and/or secretagogues, becomes limiting to glycaemic control in the late or advanced stages of disease.[2-7] Each episode of hypoglycaemia impairs the glucagon and sympathoadrenal (epinephrine) responses to subsequent hypoglycaemia, often perpetuating a cycle of recurrence.[8]

After more than 40 years of research into the mechanisms of glucagon counterregulatory failure in diabetes, evidence points to the involvement of lesser known islet hormone somatostatin (SST).[9,10] SST is secreted by δ cells of the pancreatic islets, which comprise 5% to 10% of the total islet cell mass.[11] As the putative islet gatekeeper, SST inhibits glucagon and insulin secretion via SST receptor 2 (SSTR2) and receptor 5 (SSTR5), localized to rodent α and β cells, respectively.[12,13] Pharmacological antagonism of SSTR2 augments the plasma glucagon response to both acute[14-16] and recurrent[17] hypoglycaemia in rodent models of streptozotocin (STZ) and biobreeding T1D. This restorative effect was replicated in nondiabetic rodents with overt glucagon counterregulatory failure following recurrent episodes of insulin-induced hypoglycaemia.[18] Currently in clinical trials,[19] the highly selective SSTR2 antagonist (SSTR2a), ZT-01 (Zucara Therapeutics, Toronto, ON, Canada), is emerging as a promising therapeutic for the prevention of insulin-induced hypoglycaemia in people with T1D.

Owing to relative disease prevalence, T2D, rather than T1D, now accounts for the majority of severe diabetes-related hypoglycaemic events (ie, requiring hospitalization).[20] Hypersecretion of SST, observed in T2D mouse and human islets at low glucose, offers a potential mechanism of impaired glucagon output (65%-75%) that is reversible with SSTR2a.[21] Since δ cells are electrically silenced by β cells via gap junction coupling at low glucose, mathematical modelling of δ cells predicts increasing hyperactivity and SST release under conditions of progressive β-cell death.[22] Yet, the setting in which this defect first arises remains unclear. Conflicting in vivo observations suggest that glucagon counterregulation is increased,[23] decreased[24] or unchanged[25] in chronically high-fat diet (HFD)-fed mice. However, the commonly used rodent model of HFD-induced prediabetes (pre-T2D) shows sufficient β-cell compensation to maintain normoglycaemia,[23,25] and therefore, the effect of mild hyperglycaemia on glucagon counterregulation in a setting of insulin resistance is unclear. Mild hyperglycaemia in pre-T2D is predictive of T2D risk, and β-cell exposure to mild hyperglycaemia in the transition to diabetes impairs glucose-stimulated insulin secretion,[26,27] which is most readily reversible in this early state.[28,29] This led us to question whether stimulated (ie, counterregulatory) glucagon secretion is similarly impacted by mild hyperglycaemia during the progression to diabetes and, if so, whether SSTR2 antagonism may provide a method of early correction.

We induced mild hyperglycaemia in HFD-fed rats with a single, low-dose injection of STZ followed by a period of glycaemic recovery, yielding a stable pre-T2D phenotype. We took this approach because low-dose STZ (ie, 30-40 mg/kg) induces acute hyperglycaemia and normo- or hypoinsulinaemia reminiscent of late-stage T2D in HFD-fed rats, whereas doses below this range fail to elicit significant hyperglycaemia in normal and HFD-fed male Sprague-Dawley rats.[30] The goal of this study was to characterize the counterregulatory responses of islet hormones to insulin-induced hypoglycaemia (ie, decreased insulin secretion coupled with increased glucagon secretion) in this novel pre-T2D model, with and without SSTR2a pretreatment. We hypothesized that this model would exhibit a mild defect in counterregulatory glucagon secretion that is reversible with a highly selective SSTR2a, ZT-01, to reduce hypoglycaemia exposure without modifying endogenous insulin levels or basal glycaemia.

2 | MATERIALS AND METHODS

This study was conducted in accordance with the recommendations of the Canadian Council for Animal Care guidelines and has been approved by the York University Animal Care Committee (Protocol # 2017-7).

2.1 | Animals and pre-T2D induction

Thirty (n = 30) male Sprague-Dawley rats (initial weight 200-250 g) were purchased from Envigo RMS Inc. (Indianapolis, Indiana) at an age of 8 to 9 weeks. Rats were individually housed in the York University vivarium in a 12-hour light-dark cycle with ad libitum access to food and water. Following a 1-week habituation period, 20 rats (n = 20) were randomly selected to begin an HFD (5.21 kcal/g of food), containing 60% fat, 20% carbohydrate and 20% protein (Cat# D12492; Research Diets, Inc., New Brunswick, New Jersey) for a period of approximately 7 weeks to induce obesity and insulin resistance. The remaining 10 rats served as healthy normal chow-fed controls (Cat# 5012; LabDiet, St Louis, Missouri; 13% fat, 58% carbohydrates, 29% protein; 3.5 kcal/g). After 7 weeks of HFD feeding, rats (n = 20) received a single, low-dose intraperitoneal injection of STZ (30 mg/kg; Sigma). This HFD/STZ model exhibits acute hyperglycaemia followed by a glucose recovery period from β-cell expansion that results in mild hyperglycaemia or pre-T2D.[31]

Following STZ treatment, regular drinking water was replaced with sugar water (10% w/v sucrose solution) for 2 days as a precautionary measure to prevent hypoglycaemic episodes within the first 48 hours. Hyperglycaemia was confirmed in all STZ-treated rats based on a post-absorptive whole blood glucose measurement ≥11.1 mmol/L 2 days after STZ administration. Rats that met this criterion (n = 20) remained on high-fat chow for the remainder of the

study. From this point onward, body weight, blood glucose (Contour Next glucose meter and test strips; Ascensia Diabetes Care, Mississauga, Canada), and food consumption were measured daily. Initiated 5 days after STZ treatment, basal insulin (Lantus SoloSTAR insulin glargine; Sanofi-Aventis, Bridgewater, New Jersey) was administered each evening (~6:00 PM), as needed, for 2 weeks, to help promote glycaemic recovery to the status of pre-T2D.[28,32] Therapeutic insulin was dosed based on evening (ie, pre-feeding) blood glucose measurements according to the following sliding scale: <12 mmol/L: 0 U; 12 to 15 mmol/L: 1 U; >15 mmol/L: 4 U.

2.2 | Hypoglycaemia challenges

After 2 weeks of basal insulin therapy, the rats were randomly assigned to the SSTR2a or vehicle group for the first of two hypoglycaemic challenges, conducted 1 week apart in a treatment-crossover design. Baseline glucose and hormone measurements were taken from all animals at 9:00 AM on hypoglycaemia challenge days following a controlled overnight feed to standardize food intake and preserve liver glycogen stores.[17] SSTR2a (3 mg/kg ZT-01; formulated by AdMare BioInnovations, Vancouver, BC, Canada.; supplied by Zucara Therapeutics) with vehicle (2.1% v/v glycerol in 10 mM acetate buffer pH 4.2), or vehicle alone, were then administered by subcutaneous (SC) injection 1 hour ($t = -60$ minutes) before an SC bolus injection of insulin aspart (6 U/kg NovoRapid insulin; Novo Nordisk, Bagsværd, Denmark) at $t = 0$ minutes. This dose of ZT-01 was selected because it reflects the upper limit of the dosing range tested previously in T1D rodents under hypoglycaemic clamp conditions.[16] Blood glucose was measured in duplicate via tail prick using the hand-held glucometer (described above) at $t = -60$, -30 and 0 minutes, and every 10 minutes thereafter until $t = 60$ and $t = 120$ minutes in healthy and pre-T2D rats, respectively. Saphenous vein blood samples were collected at $t = -60$, 0, 40 and 60 minutes from healthy and pre-T2D rats, and at $t = 80$ and 120 minutes, from pre-T2D rats only for subsequent hormone analysis. Most healthy rats (regardless of treatment group) reached the humane endpoint of the study (ie, signs of distress, extreme lethargy, and/or blood glucose ≤1.5 mmol/L) by 80 minutes post-insulin injection, so data collected beyond $t = 60$ minutes were excluded from analysis due to low remaining sample size. Following the second crossover challenge, all animals were anaesthetized for portal vein blood collection and then euthanized via exsanguination. Data were pooled from both hypoglycaemia challenges for analysis.

2.3 | Plasma analysis

Blood samples were collected from saphenous or portal vein bleed (terminal time point only) in potassium-EDTA coated, microvette capillary tubes (Cat # 16.444.100, Sarstedt, Canada) and centrifuged at 13523 × *g* for 5 minutes. Plasma was removed and stored in polyethylene tubes at −80°C for the subsequent quantification of glucagon (Mercodia Cat# 10-1271-01, RRID:AB_2737304) and C-peptide (Crystal Chem Cat# 90055, RRID:AB_2893130) levels using ELISA.

2.4 | Statistical analysis

Data are expressed as means ± SD, unless otherwise stated. Statistical tests were conducted against a significance criterion of $P < 0.05$ using PRISM 8 software (GraphPad, San Diego, California). Daily body weight and blood glucose levels measured throughout the study period (within-subject factor: time × between-subject factor: diabetes status); baseline (ie, pre-hypoglycaemia challenge) body weight, blood glucose, and plasma hormone levels; peak and nadir plasma hormone and nadir blood glucose levels; plasma hormone area under the curve (AUC); percent change in C-peptide levels from baseline; and portal vein glucagon levels (within-subject factor: treatment × between-subject factor: diabetes status) were analysed by two-way mixed-model analysis of variance (ANOVA), followed by Sidak post hoc tests. Blood glucose and plasma hormone concentrations measured during hypoglycaemia challenges were analysed by two-way repeated-measures ANOVA (factors: treatment × time), followed by Sidak post hoc tests. Paired *t*-tests were used to compare time to hypoglycaemia onset and blood glucose AUC between treatment groups. Probability of euglycaemia was evaluated using a log-rank (Mantel-Cox) test. The linear relationship between whole blood glucose and plasma glucagon concentration was modelled using simple linear regression and regression line slopes were analysed by two-way mixed-model ANOVA (within-subject factor: treatment × between-subject factor: diabetes status) and Sidak post hoc tests.

3 | RESULTS

3.1 | Pre-T2D model characteristics

Body weight and whole blood glucose levels, measured from the time of STZ administration until study completion, as well as body weight, whole blood glucose, and plasma hormone levels measured prior to drug/vehicle dosing on the day of experimental hypoglycaemia challenges are shown in Figure 1. Rats in the STZ/HFD group were severely hyperglycaemic after STZ treatment, reaching a peak on study day 3 (22.5 ± 6.8 mmol/L vs. 5.1 ± 0.4 mmol/L in healthy rats), and gradually reverting towards pre-T2D over the glycaemic recovery period (Figure 1A). Mean daily basal insulin requirement diminished throughout the study period, from 3.3 ± 1.6 U/d on day 1 of insulin maintenance (study day 6) to 0.2 ± 1.0 U/d approximately 3 weeks later (study day 27; data not shown). At the time of hypoglycaemia challenges, only one of 17 HFD/STZ rats required basal insulin therapy (data not shown). Body weight remained stable in the normal-chow-fed healthy controls throughout the 4-week study period, whereas HFD-fed rats exhibited rapid weight loss after STZ treatment, reaching a nadir by day 3 and recovering 1 week later (Figure 1B). Body weight plateaued with continued HFD feeding for the remainder of the study (Figure 1B).

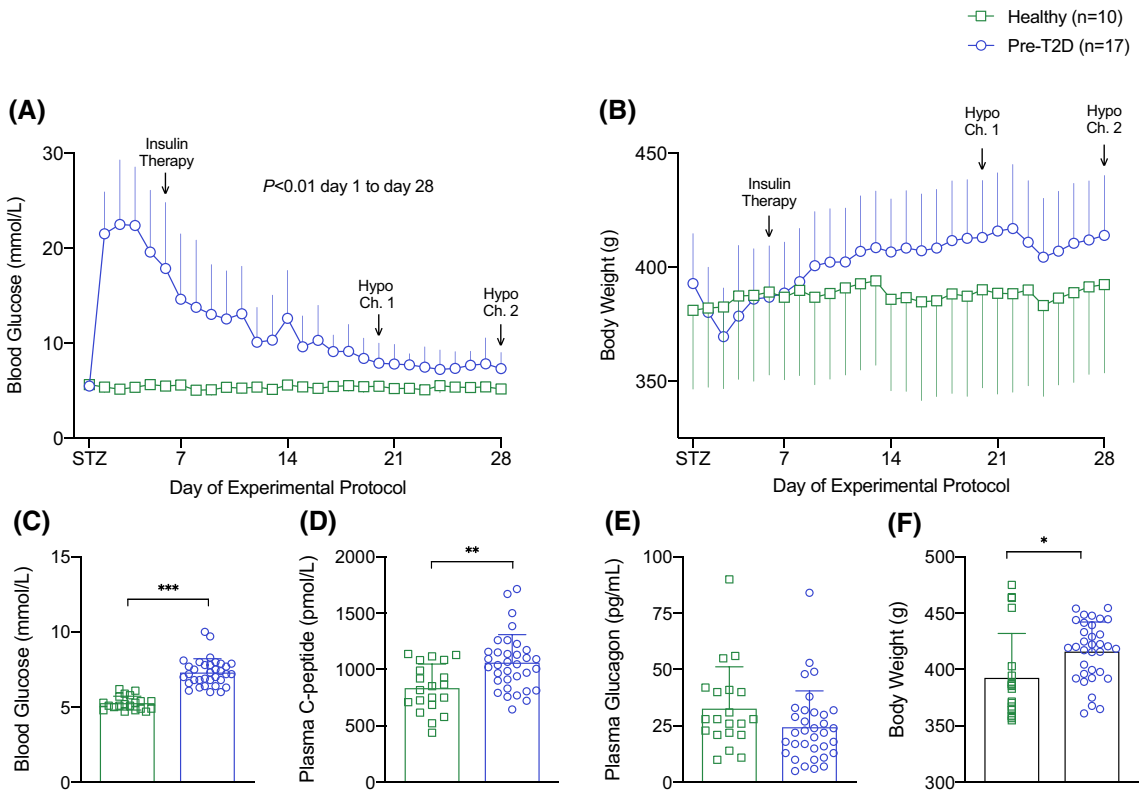

FIGURE 1 Prediabetes (pre-T2D) model characteristics. (A) Whole blood glucose concentration and (B) body weight measured from the time of STZ administration (day 1) to the second hypoglycaemia challenge (study completion; day 28). The initiation of daily (blood glucose-dependent) insulin therapy is indicated on day 6. Pooled baseline measurements of (C) whole blood glucose concentration, (D) plasma C-peptide concentration, (E) plasma glucagon concentration and (F) body weight on hypoglycaemia challenge days 1 and 2. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. All data are means ± SD. Hypo Ch., hypoglycaemia challenge; STZ, streptozotocin

Mean baseline measurements in pre-T2D rats did not differ significantly between experimental days (ie, hypoglycaemia challenges) 1 and 2, separated by a 1-week washout period (ie, body weight [415 ± 26 g vs. 417 ± 28 g], whole blood glucose [7.4 ± 1.1 mmol/L vs. 7.3 ± 0.6 mmol/L], plasma glucagon [23 ± 15 pg/mL vs. 24 ± 19 pg/mL] and plasma C-peptide [1038 ± 230 pmol/L vs. 1111 ± 281 pmol/L] levels), so data from both experiments were pooled for analysis. Collectively, three animals met the diagnostic criteria for overt T2D by a random glucose measurement in humans (≥11.1 mmol/L)[33] on challenge day 1 (24.4 mmol/L) and day 2 (13.0 and 11.9 mmol/L, respectively), so their data were excluded from all analyses, reducing the sample size of the pre-T2D group to n = 17.

At the time of the hypoglycaemia challenges (ie, 2 and 3 weeks after STZ treatment), a mild hyperglycaemic phenotype was observed in our pre-T2D rat model, as measured in the post-absorptive state after a controlled overnight feed and without basal insulin therapy (pre-T2D: 7.4 ± 1.2 mmol/L vs. healthy: 5.3 ± 0.5 mmol/L; $P < 0.001$ [Figure 1C]). In combination with mild baseline hyperglycaemia, plasma C-peptide levels were also increased relative to healthy controls (pre-T2D: 1061 ± 249 pmol/L vs. healthy: 836 ± 211 pmol/L; $P < 0.01$ [Figure 1D]), while plasma glucagon concentration was unchanged (pre-T2D: 24 ± 16 pg/mL vs. healthy: 33 ± 19 pg/mL [Figure 1E]). Finally, body weight was modestly yet significantly higher ($P < 0.05$) in pre-T2D (416 ± 26 g) versus healthy control rats (393 ± 39 g [Figure 1F]).

3.2 | Hypoglycaemia challenges

3.2.1 | Blood glucose

In the healthy rats, blood glucose levels were not significantly different between vehicle and SSTR2a groups at baseline or any subsequent sampling timepoint (Figure 2A). Blood glucose levels remained unchanged from basal values for 1 hour after SSTR2a or vehicle treatment (ie, $t = 0$ minute) and fell uniformly in both groups after insulin bolus, reaching similar hypoglycaemic nadirs by $t = 60$ minutes (healthy vehicle:1.6 ± 0.3 mmol/L vs. healthy SSTR2a: 1.4 ± 0.3 mmol/L [Figure 2A]). Time to hypoglycaemia onset (healthy vehicle: 27 ± 8 minutes vs. healthy SSTR2a: 24 ± 7 minutes [-Figure 2B]) and probability of euglycaemia (≥4.0 mmol/L) over time (Figure 2C) were unaffected by SSTR2a treatment in healthy rats.

In the pre-T2D rats, blood glucose levels were identical between treatment groups at baseline (vehicle: 7.3 ± 0.9 mmol/L vs. SSTR2a: 7.3 ± 0.9 mmol/L [Figure 2D]). However, glycaemia rose by 1.5 mmol/L from baseline (7.3 ± 0.9 mmol/L to 8.8 ± 2.1 mmol/L;

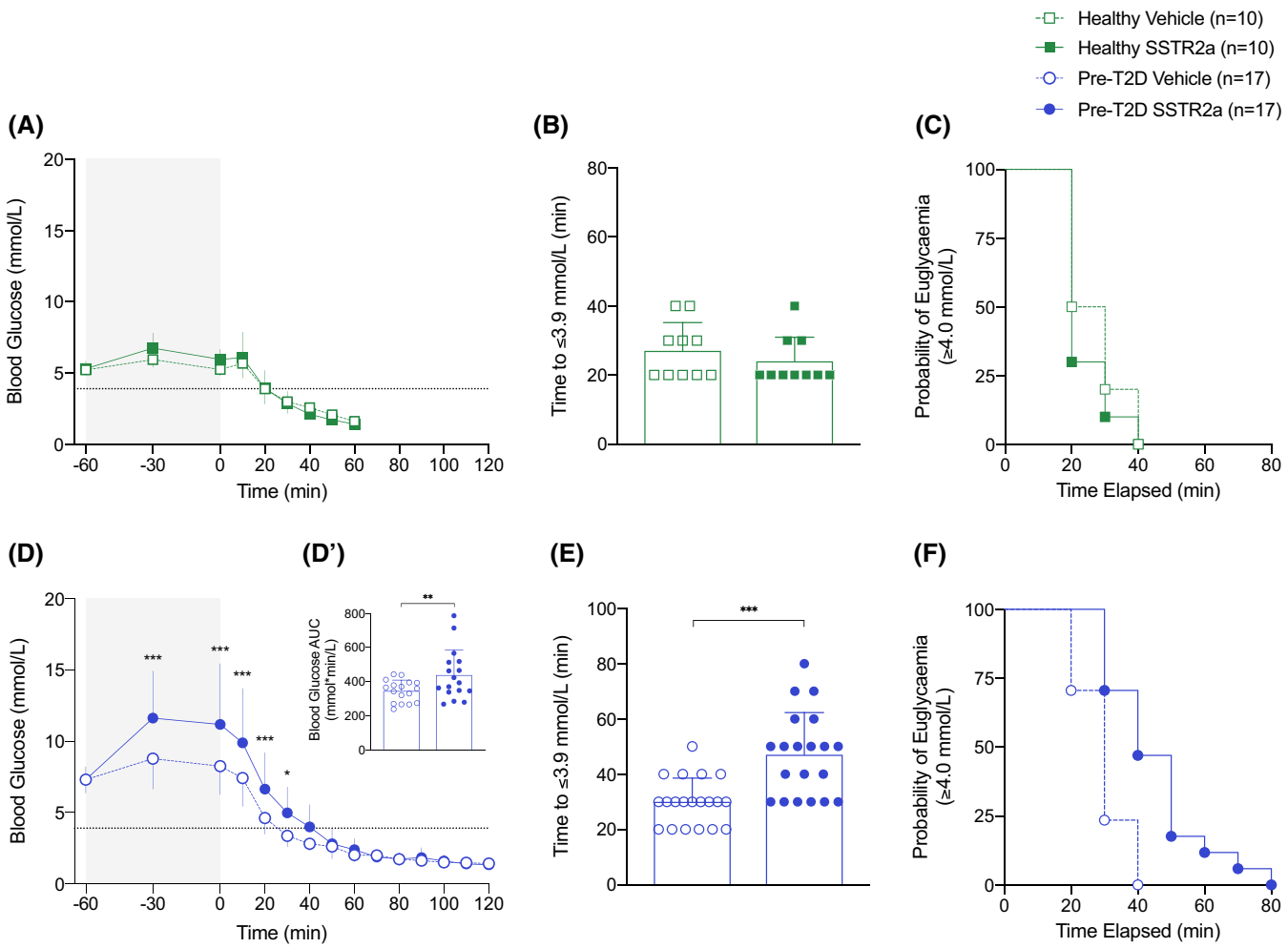

FIGURE 2 Whole blood glucose responses to insulin-induced hypoglycaemia in (A) healthy and (B) prediabetic (pre-T2D) rats with or without somatostatin receptor 2 antagonist (SSTR2a). Grey shaded region indicates the period of basal monitoring before hypoglycaemia induction at $t = 0$ minutes. Time from insulin administration to the onset of clinical hypoglycaemia (≤3.9 mmol/L) in (C) healthy and (D) pre-T2D rats. (D') Blood glucose area under the curve (AUC) from $t = 0$ to $t = 120$ minutes in pre-T2D rats. Survival curve comparing the proportion of (E) healthy and (F) pre-T2D rats ($P < 0.001$ SSTR2a vs. vehicle) remaining euglycaemic (≥4.0 mmol/L) after hypoglycaemia induction. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ between treatment groups. All data are means ± SD

nonsignificant) in the vehicle group and by 4.3 mmol/L (7.3 ± 0.9 mmol/L to 11.6 ± 3.3 mmol/L; $P < 0.001$) in the SSTR2a group within 30 minutes ($t = -30$ minutes) of treatment (Figure 2D). Blood glucose levels remained significantly higher in the SSTR2a versus the vehicle group from 30 minutes post-SSTR2a/vehicle administration ($t = -30$ minutes) until 30 minutes post-insulin administration ($t = 30$ minutes, $P < 0.001$ for $t = -30$, 0, 10 and 20 minutes; $P < 0.05$ for $t = 30$ minutes [Figure 2D]). Blood glucose nadir during hypoglycaemia was unaffected by treatment (pre-T2D vehicle: 1.4 ± 0.3 mmol/L vs. T2D SSTR2a: 1.3 ± 0.4 mmol/L [Figure 2D]). Blood glucose AUC after $t = 0$ minutes was 1.3-fold higher ($P < 0.001$) in the pre-T2D-SSTR2a group (498 ± 203 mmol*min/L) versus the pre-T2D-vehicle group (375 ± 133 mmol*min/L [Figure 2D]).

The onset of hypoglycaemia, defined by the American Diabetes Association Workgroup on Hypoglycaemia as blood glucose ≤3.9 mmol/L in humans with T1D or T2D,[34] was delayed by 15 ± 12 minutes ($P < 0.001$) in the T2D-SSTR2a group (44 ± 13 minutes) compared with the pre-T2D-vehicle group (28 ± 7 minutes [Figure 2E]). A comparison of survival curves in Figure 2F revealed a lower proportion of hypoglycaemic pre-T2D rats treated with SSTR2a as compared with vehicle ($P < 0.001$) throughout the challenge period. Notably, 18% (n = 3/17) of vehicle-treated versus 65% (n = 1/17) of SSTR2a-treated rats remained euglycaemic (≥4.0 mmol/L) 30 minutes after insulin administration, and 100% of the rats in each group reached hypoglycaemia by 40 and 70 minutes, respectively (Figure 2F).

Figure 3 illustrates the effects of SSTR2a treatment on depth of hypoglycaemia (Figure 3A) and hyperglycaemia (Figure 3B) in pre-T2D rats. Blood glucose AUC ≤3.9 mmol/L was 25% lower ($P < 0.01$) with SSTR2a (128 ± 44 mmol*min/L) versus vehicle (171 ± 43 mmol*min/L) treatment (Figure 3A). Blood glucose AUC ≥11.1 mmol/L was not significantly higher in the SSTR2a group (vehicle: 4 ± 10 mmol min/L vs. SSTR2a: 77 ± 160 mmol*min/L [Figure 3B]; however, 30% (n = 3/10) versus 63%

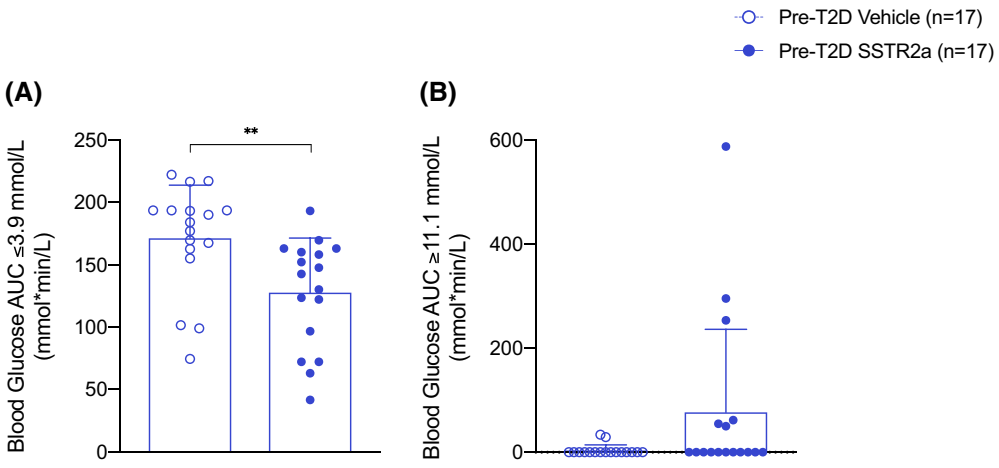

FIGURE 3 Whole blood glucose area under the curve (AUC) of prediabetes (pre-T2D) rats (A) ≤3.9 mmol/L and (B) ≥11.1 mmol/L with or without somatostatin receptor 2 antagonist (SSTR2a). $^{**}P < 0.01$. All data are means ± SD

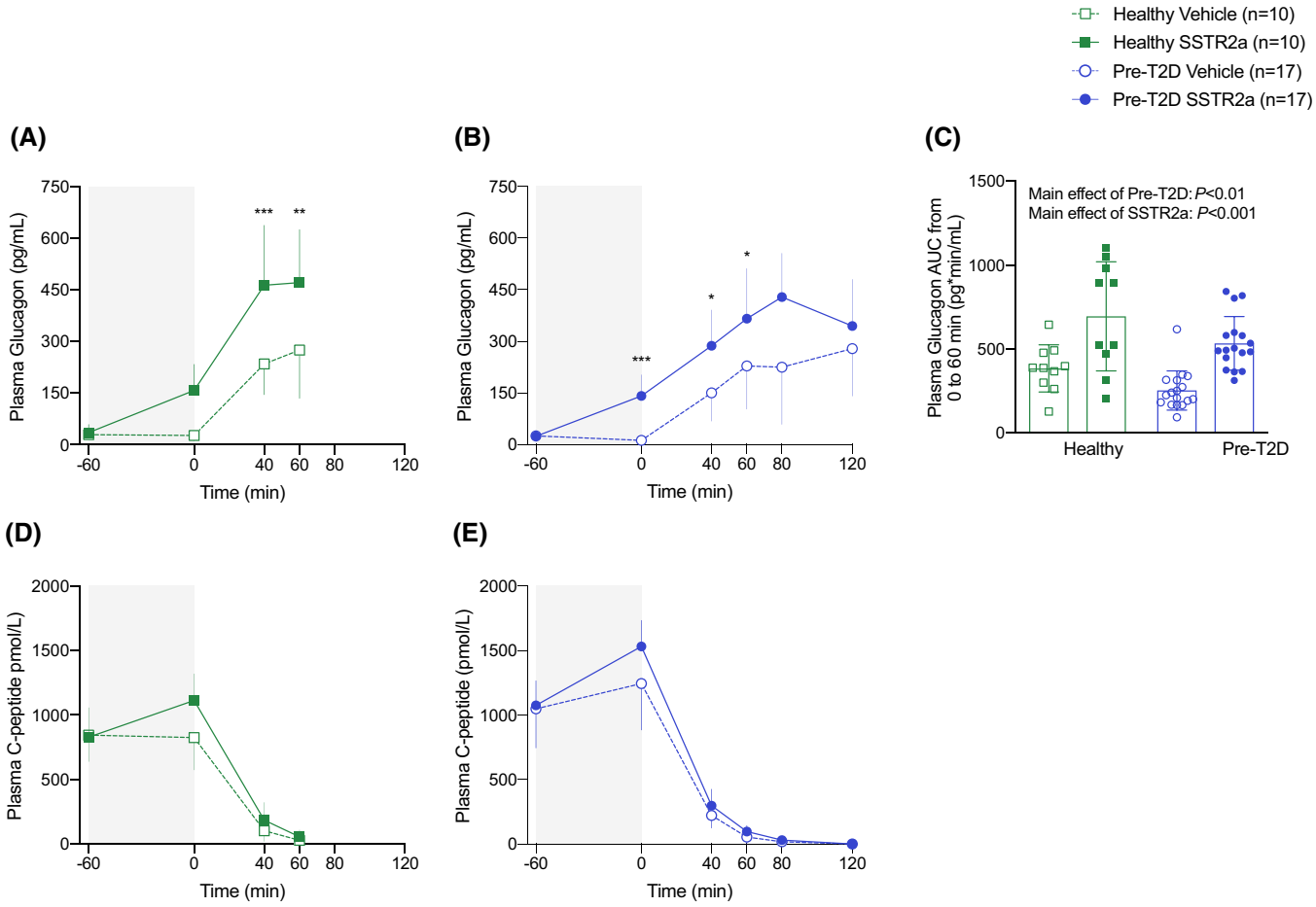

FIGURE 4 Plasma glucagon responses to insulin-induced hypoglycaemia in (A) healthy, and (B) prediabetes (pre-T2D) rats with or without somatostatin receptor 2 antagonist (SSTR2a). (C) Plasma glucagon area under the curve (AUC) from $t = 0$ minutes to $t = 60$ minutes in all conditions. Plasma C-peptide concentration in (D) healthy and (E) pre-T2D rats. Grey shaded region indicates period between SSTR2a/vehicle dosing and bolus insulin administration. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ between treatment groups. All data are means ± SD

(n = 9/17) of pre-T2D rats reached a blood glucose concentration of ≥11.1 mmol/L after treatment with vehicle versus SSTR2a, respectively (chi-squared test, $P = 0.25$). Hyperglycaemia was defined by a lower limit of 11.1 mmol/L for this outcome because it reflects the clinical threshold for T2D diagnosis from a random blood glucose measurement.[33]

3.2.2 | Plasma hormones

Plasma glucagon and C-peptide concentrations measured during iatrogenic hypoglycaemia challenge are shown in Figure 4. Plasma glucagon levels did not differ significantly between the two treatment

groups of healthy (ie, control) rats at baseline (vehicle: 30 ± 13 pg/mL vs. SSTR2a: 35 ± 24 pg/mL [Figure 4A]). In healthy rats, glucagon levels rose 4.6-fold from baseline 1 hour after SSTR2a treatment (t = 0 minutes: 158 ± 76 pg/mL vs. t = −60 min: 35 ± 24 pg/mL; P < 0.05 [Figure 4A]). This increase was not significant relative to vehicle-control levels (t = 0 minutes: 27 ± 15 pg/mL), which remained stable from baseline (t = −60 minutes: 29 ± 12 pg/mL [Figure 4A]). In response to hypoglycaemia induction by insulin aspart bolus, plasma glucagon levels were significantly elevated for 60 minutes in the healthy-SSTR2a versus the healthy-vehicle group (time × treatment: P < 0.01 [Figure 4A]). In the pre-T2D rats, plasma glucagon levels did not differ significantly between treatment groups at baseline (vehicle: 25 ± 20 pg/mL vs. SSTR2a: 24 ± 12 pg/mL [Figure 4B]). Plasma glucagon levels were approximately 12-fold higher (P < 0.001) within 1 hour of SSTR2a treatment (vehicle: 12 ± 5 pg/mL vs. SSTR2a: 141 ± 62 pg/mL) and remained significantly elevated for 60 minutes post-insulin administration (P < 0.05 for t = 40 and 60 minutes [Figure 4B]).

A comparison of plasma glucagon responses across all four groups within 60 minutes of insulin administration is shown in Figure 4C. The plasma glucagon response to hypoglycaemia (as measured by glucagon AUC) was 1.4-fold lower in pre-T2D (394 ± 198 pg*min/mL) versus healthy (540 ± 291 pg*min/mL) rats, independent of treatment (main effect of diabetes status: P < 0.01), and approximately twofold higher with STSR2a (595 ± 237 pg min/mL) versus vehicle (302 ± 137 pg*min/mL) treatment, independent of diabetes status (main effect of treatment: P < 0.001 [Figure 4C]). Peak plasma glucagon concentration was approximately 1.5-fold higher with SSTR2a (477 ± 124 pg/mL) versus vehicle (321 ± 134 pg/mL) treatment, independent of diabetes status (main effect of treatment: P < 0.001) but was not significantly different between healthy and pre-T2D groups (data not shown).

Baseline circulating C-peptide levels were higher in pre-T2D (1061 ± 249 pmol/L) as compared with healthy rats (836 ± 212 pmol/L; main effect of diabetes status: P < 0.01 [Figure 1D]). SSTR2a had no significant effect on circulating C-peptide levels under basal or hypoglycaemic conditions in healthy (Figure 4D) or pre-T2D (Figure 4E) rodents. However, peak C-peptide levels and plasma C-peptide AUC between t = −60 and 0 minutes were both approximately 1.3-fold higher in pre-T2D (peak: 1380 ± 309 pmol/L; AUC: 72 935 ± 14 441 pmol*min/L) versus healthy rats (peak: 1048 ± 209 pmol/L; AUC: 54 151 ± 9814 pmol*min/L), independent of treatment (main effect of diabetes status: P < 0.001 for both), and 1.1 to 1.2-fold higher with SSTR2a (peak: 1383 ± 272 pmol/L; AUC: 68 600 ± 11 983 pmol min/L) versus vehicle (peak: 1131 ± 315 pmol/L; AUC: 61 617 ± 18 285 pmol*min/L), independent of diabetes status (main effect of treatment: P < 0.01 and P < 0.05 for peak and AUC, respectively; data not shown). Bolus insulin administration at t = 0 minutes triggered a decline in plasma C-peptide levels over 40 minutes in all groups, yielding a comparable reduction from baseline across all four groups (healthy-vehicle group: 87% ± 6%; healthy-SSTR2a group: 85% ± 8%; pre-T2D-vehicle group: 79% ± 9%; pre-T2D-SSTR2a group: 81% ± 8% [Figure 4D,E]).

Portal vein glucagon concentration, measured at the terminal timepoint in hypoglycaemia, did not differ significantly across the four conditions (healthy-vehicle group: 142 ± 211 pg/mL; healthy-SSTR2a group: 116 ± 162 pg/mL; pre-T2D-vehicle group: 89 ± 85 pg/mL; pre-T2D-SSTR2a group: 62 ± 63 pg/mL; data not shown), while C-peptide was undetectable in the portal vein across conditions at terminal hypoglycaemia.

The effects of pre-T2D and SSTR2a treatment on the relationship between whole blood glucose and plasma glucagon levels after insulin overdose are illustrated in Figure 5. For each group, average blood glucose level and glucagon level at baseline and each sampling timepoint after insulin bolus (t = −60, 40, 60 or 80 minutes) were linearly correlated (R^2 > 0.94 for each condition [Figure 5A]). Regression line slopes, representing the increase in plasma glucagon concentration (pg/mL) per 1-mmol/L drop in blood glucose concentration, were significant (slope >0) for all conditions (Figure 5A). A comparison of slopes for each rat in each group (Figure 5A') showed increased glucagon responsiveness to insulin-induced hypoglycaemia with SSTR2a versus vehicle (glucagon increase of 88 ± 43 vs. 52 ± 35 pg/mL per 1-mmol/L drop in glucose) treatment, independent of diabetes status (main effect of treatment: P < 0.001), and attenuated glucagon responsiveness in pre-T2D versus healthy rats (glucagon increase of 56 ± 30 vs. 97 ± 50 pg/mL per 1-mmol/L decline in glucose), independent of treatment (main effect of diabetes status: P < 0.001 [Figure 5A']).

4 | DISCUSSION

In this study, we developed a novel rat model of pre-T2D that exhibited mild hyperglycaemia and moderate insulin resistance, to better understand the combined effects of these pre-T2D hallmarks on glucagon counterregulation to hypoglycaemia, with and without SSTR2 antagonism. We report a mild attenuation in the magnitude of the glucagon response, as well as glucagon responsiveness (ie, α-cell sensitivity) to hypoglycaemia development following insulin bolus challenge in our novel pre-T2D model. Pretreatment with SSTR2a increased the plasma glucagon response during hypoglycaemia by all measures (peak concentration, AUC, and responsiveness) in both healthy and pre-T2D rats. This glucagon response was associated with approximately 30 minutes' elevation in blood glucose levels compared with vehicle controls and 15 minutes' delay in the onset of hypoglycaemia, which were not observed in healthy rats. Consistent with evidence from SST knockout islets,[10] SSTR2a treatment in the present study did not affect the C-peptide "switch-off" response to insulin-induced hypoglycaemia in healthy or pre-T2D animals. This finding confirms antagonist selectivity for SSTR2 (expressed by α cells) over SSTR5 (expressed by rodent β cells).[35] Collectively, these data suggest that SSTR2a treatment may have therapeutic applications for hypoglycaemia prevention in more advanced stages of insulin-deficient T2D.

Recent findings suggest that the suppression of SST secretion from islet δ cells may act as a permissive signal for counterregulatory glucagon release, which is compromised in T2D.[21] In one study, islets from hyperglycaemic Fh1βKO T2D mice showed a sixfold increase in SST secretion at 1 mmol/L glucose relative to healthy control islets,

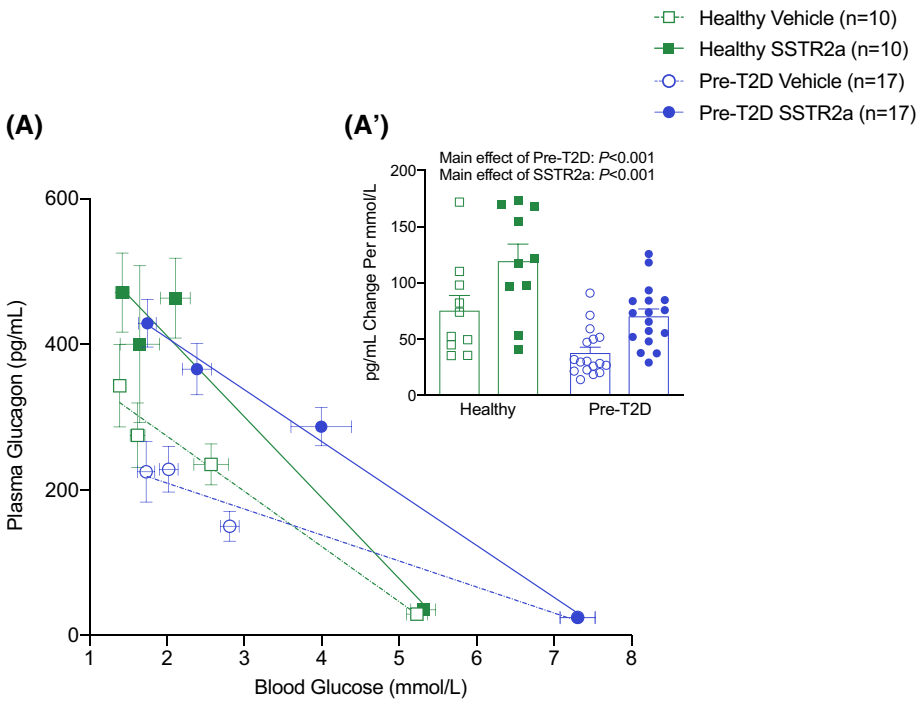

FIGURE 5 Relationship between plasma glucagon and whole blood glucose levels in healthy and prediabetes (pre-T2D) rats after insulin overdose with or without somatostatin receptor 2 antagonist (SSTR2a). (A) Regression line fit to grouped timepoint data (t = −60, 40, 60 and 80 minutes) for each condition. (A') Comparison of regression line slopes across conditions. All data are means ± SEM

which correlated with a >75% reduction in glucagon secretion.[21] Consistent with elevated SST tone in T2D islets, application of a SSTR2a at 1 mmol/L glucose raised glucagon secretion by 143% ± 11% in Fh1βKO islets compared with 13% ± 14% in control islets.[21] Alternatively, reports of a decrease in SST secretion[21,36] and acquired α-cell resistance to SST[36] at higher glucose concentrations may help to explain the hyperglucagonaemia in T2D. These findings were replicated in the perfused pancreas and isolated islets of HFD-fed mice across a wider glucose range (1, 6 and 15 mmol/L glucose).[23] Accordingly, SSTR2 antagonism had little to no effect on glucagon secretion in HFD islets, or in T2D islets at high glucose, unlike the marked stimulatory effect observed in control islets.[21,23] However, it is important to note that these in situ and in vitro responses were observed under conditions of low glucose alone—that is, without the inhibitory effect of exogenous insulin on counterregulatory glucagon secretion.[37] When measured in HFD-fed mice following bolus insulin challenge in vivo, the counterregulatory glucagon response was only increased relative to controls when insulin was dosed according to lean and not total body mass.[23] In the present study, the stimulatory effect of SSTR2 antagonism on plasma glucagon levels was similar in pre-T2D and healthy rats during basal glycaemia and insulin-induced hypoglycaemia, suggesting that SST signalling may be relatively normal in this model compared to more advanced stages of T2D.[21] This outcome further highlights the potential for SSTR2a to induce transient hyperglucagonaemia in the absence of hypoglycaemia at the present dose in T2D.

Islet β cells undergo morphological (ie, β-cell expansion) and functional adaptations to maintain normoglycaemia in a setting of insulin resistance.[38] Over time, increased insulin demand leads to gradual β-cell de-differentiation, and the loss of adequate compensation precipitates the onset of pre-T2D, characterized by impaired glucose tolerance and impaired fasting glucose.[38,39] Hyperinsulinaemia, which persists from pre-T2D through early-stage T2D, is eventually normalized (relative to controls) with advancing β-cell failure, resulting in the onset of late-stage T2D.[40] Despite phenotypic heterogeneity within and between clinical T2D study samples (ie, mean disease and treatment duration, treatment intensity and modality, etc.), basal hypoinsulinaemia relative to nondiabetic control subjects (ie, late-stage T2D) has emerged as a clinical surrogate of defective glucose counterregulation in T2D.[8,41,42] Here, we report a mild defect in counterregulatory glucagon secretion during insulin-induced hypoglycaemia in pre-T2D rats, despite basal hyperinsulinaemia. These observations suggest that this progressive α-cell defect may begin to develop in a state of relative insulin deficiency (relative to ambient blood glucose concentration)—that is, before the onset of overt or absolute insulin deficiency (relative to healthy controls), as previously speculated.

A robust SSTR2a-induced increase in plasma glucagon levels, observed before and after hypoglycaemic induction in healthy and pre-T2D rats, only amounted to a detectable increase in blood glucose levels in the pre-T2D group. We propose that the basal hyperglycaemic insult posed by SSTR2 antagonism in the healthy group was offset by endogenous insulin action (based on increases in peak plasma C-peptide levels and plasma C-peptide AUC before bolus insulin challenge) to preserve euglycaemia. This increase in endogenous insulin levels, observed after SSTR2a treatment but before insulin bolus in both healthy and pre-T2D rats, may also reflect paracrine stimulation by increased levels of intra-islet glucagon.[43] After insulin bolus, SSTR2a afforded no protection against hypoglycaemia in healthy rats, perhaps because the glucagon response was outmatched by the dose of exogenous insulin used. This outcome in healthy rats was similar to that of a previous study, in which SSTR2a treatment in STZ-T1D rats reduced the dependence on glucose infusion for the maintenance of

clamped hypoglycaemia in the presence of low- but not high-dose insulin, despite a complete normalization of glucagon levels at both doses.[15] We suspect that in a setting of peripheral insulin resistance and relative insulin deficiency, the hyperglycaemic effect of the SSTR2a could not be offset by the actions of endogenous or exogenous insulin, delaying hypoglycaemia onset in our pre-T2D model.

This study had several limitations. First, it tested a single low dose of ZT-01, and therefore, future studies are necessary to determine the minimum effective dose of this particular SSTR2a that does not aggravate basal hyperglycaemia in this or other models of pre-T2D and T2D. Second, portal vein hormones were measured only at hypoglycaemic challenge completion; however, a measurable effect of SSTR2a on portal hormone levels may have dissipated by this time. Regular portal vein sampling from pre-implanted catheters may offer more descriptive insights into islet hormone responses in vivo (including response rates and glycaemic thresholds). Finally, non-normalized blood glucose levels in our pre-T2D rats at baseline, compounded by insulin resistance in this model, extended the time to terminal hypoglycaemia by 60 minutes relative to healthy rats. This limited our comparison of total hormone responses to hypoglycaemia (ie, AUC analyses) between healthy and pre-T2D rats.

In summary, the glucagon counterregulatory response to insulin-induced hypoglycaemia was mildly impaired in this novel rat model of pre-T2D that exhibits insulin resistance and mild hyperglycaemia. Nonetheless, SSTR2a treatment augmented plasma glucagon levels after bolus insulin overdose and delayed the onset of hypoglycaemia in prediabetic rodents without affecting the plasma C-peptide response in this rodent model. However promising, these treatment outcomes were not low-glucose-dependent, suggesting that SST may be important for regulating glucagon secretion under basal (postprandial) and hypoglycaemic conditions in vivo. Consequently, SSTR2a may aggravate basal hyperglycaemia at the current dose in a setting of relative insulin deficiency and peripheral insulin resistance. Collectively, these discoveries stand to advance our understanding of the paracrine mechanisms governing α-cell behaviour in health and disease, which may be vital to improving therapeutic options for individuals living with early- and late-stage T2D.

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CONFLICT OF INTEREST STATEMENT

Richard Liggins is an employee of Zucara Therapeutics who provided the SSTR2a. Michael C. Riddell serves on the scientific advisory board for Zucara Therapeutics. All other authors declare that there are no relationships or activities that might bias, or be perceived to bias, their work.

PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1111/dom.15002.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Emily G. Hoffman https://orcid.org/0000-0003-1616-6454
Michael C. Riddell https://orcid.org/0000-0001-6556-7559

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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