Somatostatin Receptor Type 2 Antagonism Improves Glucagon Counterregulation in Biobreeding Diabetic Rats

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Impaired counterregulation during hypoglycemia in type 1 diabetes (T1D) is partly attributable to inadequate glucagon secretion. Intra-islet somatostatin (SST) suppression of hypoglycemia-stimulated α-cell glucagon release plays an important role. We hypothesized that hypoglycemia can be prevented in autoimmune T1D by SST receptor type 2 (SSTR2) antagonism of α-cells, which relieve SSTR2 inhibition, thereby increasing glucagon secretion. Diabetic biobreeding diabetes-prone (BBDP) rats mimic insulin-dependent human autoimmune T1D, whereas nondiabetic BBDP rats mimic prediabetes. Diabetic and nondiabetic rats underwent a 3-h infusion of vehicle compared with SSTR2 antagonist (SSTR2a) during insulin-induced hypoglycemia clamped at 3 ± 0.5 mmol/L. Diabetic rats treated with SSTR2a needed little or no glucose infusion compared with untreated rats. We attribute this effect to SSTR2a restoration of the attenuated glucagon response. Direct effects of SSTR2a on α-cells was assessed by resecting the pancreas, which was cut into fine slices and subjected to perifusion to monitor glucagon release. SSTR2a treatment enhanced low-glucose–stimulated glucagon and corticosterone secretion to normal levels in diabetic rats. SSTR2a had similar effects in vivo in nondiabetic rats and promoted glucagon secretion from nondiabetic rat and human pancreas slices. We conclude that SST 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. ***Diabetes*** **62:2968–2977, 2013**

In type 1 diabetes (T1D), 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 counterregulation that is largely, but not solely, attributed to "glucose blindness" of pancreatic islet α-cells, which are unable to secrete glucagon to mobilize glucose from the liver (2). Recent focus on the mechanism for α-cell glucose blindness has centered on the role of D-cell somatostatin (SST), culminating in the concept of a defective SST "switch-off" (3). In T1D, because of β-cell destruction, hypoglycemia could no longer induce the decrement reduction in insulin release, a required signal for α-cells to release glucagon during hypoglycemia (4,5). SST from adjacent D-cells is thought to play a minor role in inhibiting α-cells in nondiabetic animals and humans because of this dominant inhibitory role of insulin. In the absence of intra-islet insulin in T1D, however, SST paracrine inhibition of the α-cell becomes manifest (3). As further support for a dominating role for the inhibitory action of SST on D-cell glucagon release, it recently was shown that tolbutamide inhibition of glucagon secretion also was largely attributable to the SST released from D-cells (6). It would seem that this SST inhibition of glucagon secretion is accentuated in T1D. It has been known for some time that exogenously added SST could inhibit pancreatic glucagon release (7). This was confirmed in SST receptor type 2 (SSTR2) knockout 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 SST, pancreatic prosomatostatin mRNA, and SST protein levels are increased (10,11).

The most convincing evidence has been the effects of SST receptor agonists (12) and antagonists (13) on glucagon secretory response, which identified SSTR2 as the putative receptor on α-cells, as confirmed by studies on SSTR2 knockout mice (8). This led to the development of specific SSTR2 antagonists (SSTRas) such as PRL-2903 (14), shown to antagonize endogenous SST, resulting in enhancement of arginine-stimulated glucagon secretion from perfused pancreata and perifused islets of nondiabetic rats (13). PRL-2903 is selective for SSTR2 over SSTR3 and SSTR5 by 10-fold 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 counter-regulatory 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 T1D, which is an autoimmune disorder. Furthermore, this model does not require insulin treatment, whereas human T1D does. In this study, we have used the insulin-requiring biobreeding (BB) diabetic-prone (BBDP) rat as an authentic autoimmune model of human

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T1D (16,17) to test the therapeutic actions of SSTR2 blocker PRL-2903. BBDP rats are prediabetic before diabetes develops (18,19), and their islet morphology shows infiltration with inflammatory cells (insulinitis) before the disappearance of β-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 β-cell destruction and the prediabetic state with insulinitis (used in this study as control), BB rat α-cell SSTR signaling could be perturbed but could be corrected by the SSTR2a. With use of the hyperinsulinemic hypoglycemic clamp technique, PRL-2903 restored glucagon secretion in the diabetic BBDP rats. Central (hypothalamic) and peripheral counter-regulatory pathways (catecholamines, corticosterone) are also influenced by SST, and they also affect glucagon secretion (10,20), and thus they could confound the interpretation of the direct actions of PRL-2903 on α-cells. Because islets could not be reliably isolated from T1D 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 BBDP/Wor rats (Biomere, Worcester, MA) were housed in a sterile animal facility, fed a standard diet, and maintained on a 12-h/12-h day/night cycle. Age-matched BBDP rats that did not become diabetic at an age range of 60–150 days were used as controls. BBDP rats that became diabetic (random blood glucose >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 the Canadian Council on Animal Care Standards and were 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 Toronto General Hospital, with preoperative written consent obtained from source patients. The surgical pathology laboratory, after assessing the resected tissue, immediately placed a normal portion in extracellular solution; our laboratory was then called to retrieve the specimen to process. Experiments were 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.

SSTR2a. The SSTR2a peptide, PRL-2903, was synthesized by D.H.C. and was 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 the following four groups: diabetic vehicle (n = 8), diabetic plus SSTR2a (n = 8), nondiabetic vehicle (n = 5), and nondiabetic plus SSTR2a (n = 6). The surgery and clamp procedures were performed as described in our previous study of STZ-treated rats (10). Surgery was performed 7 days before the clamp experiments; for diabetic rats, surgery was performed 14 days after insulin pellet implantation. Rats were fasted overnight before the clamp study and then weighed. Under general anesthesia 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 h with great care to avoid any stress throughout the clamp procedure. After obtaining baseline blood samples at −60 min, the rats underwent a 3-h infusion of vehicle (1% acetic acid in 0.9% saline) or SSTR2a (1,500 nmol/kg/h) at an infusion rate of 1 mL/h with a digital syringe infusion pump (Harvard Apparatus, Holliston, MA). To induce hypoglycemia to a target level of 3 ± 0.5 mmol/L, insulin infusion at the constant rate of 20–50 mU/kg/min together with variable rates of glucose infusion was started at 0 min. During the clamp, blood samples were obtained from the carotid catheter every 10 min: one set into capillary tubes coated with Kalium-EDTA (Microvette CB 3000; Sarstedt Montreal, QC, Canada) and immediately centrifuged (room temperature), with plasma glucose levels then determined by glucose analyzer (Analox Glucose analyzer; Analox Instruments, London, U.K.), and another set for hormone measurements.

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

Plasma hormone measurements. Blood samples were obtained at −60 min, then every 30 min from 0 min in ice-chilled tubes containing 5 μL of 100 mmol/L EDTA solution and 30 kallikrein inhibitor units of aprotinin (APR600; BioShop Canada, Burlington, ON, Canada), followed by centrifugation at 12,000 rpm at 4°C to separate the plasma. The plasma was aliquoted and immediately frozen on dry ice and transferred to −80°C to await hormone measurements for glucagon (radioimmunoassay kit; EMD Millipore, Darmstadt, Germany), corticosterone (ELISA kit; ALPCO Diagnostics, Salem, NH), norepinephrine/epinephrine (2-Cat Plasma ELISA kit; Labor Diagnostica Nord GmbH & Co. KG, Nordhorn, Germany), and SST (extraction-free rat SST-14 enzyme immunoassay kit; Bachem Group, Bubendorf, Switzerland).

Total pancreatic glucagon and SST 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 1 mol/L pH 7.5 Tris buffer. SST and glucagon levels were determined as described 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). On euthanization, the rat abdominal cavity was opened and the common bile duct was identified, clamped, and injected with 3 mL 37°C low-melting 1.9% agarose gel (cat. no. 15517-022; Invitrogen, Camarillo, CA). The pancreas was resected, cut into smaller pieces, embedded in 1.9% agarose gel, and cooled to become solidified. The tissue blocks were placed in Carbogen (5% CO_2, 95% O_2) bubbled ice-cold extracellular solution and sliced into 4 × 4 mm, 140-μm-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 $MgCl_2$, 2 $CaCl_2$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 2 Na pyruvate, 0.25 ascorbic acid, 3 *myo*-inositol, 6 lactic acid, and 7 glucose. Human pancreas slices were similarly prepared after embedding into the agarose gel.

Ten 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 Krebs-Ringer bicarbonate buffer solution containing (in mmol/L) 135 NaCl, 3.6 KCl, 0.50 NaH_2PO_4, 0.5 $MgCl_2$, 1.5 $CaCl_2$, 2 $NaHCO_3$, 10 HEPES, and 1 g/L BSA, at a flow rate of 1 mL/min, and samples were collected at 1-min intervals. As indicated in the study protocols, the perifusion solution would contain 1 or 7 mmol/L glucose, with or without SSTR2a (PRL-2903, 30 μmol/L) or arginine (20 mmol/L). Each study was preceded with a 20-min equilibration period at 7 mmol/L glucose. The BB rat pancreas slice study was performed in the following four groups: diabetic, diabetic plus SSTR2a, nondiabetic, and nondiabetic plus SSTR2a. Samples were collected at 1-min intervals into ice-chilled tubes containing 1,000 kallikrein inhibitor units of aprotinin, frozen, and stored at −80°C to await 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 set-up 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 performed with monoclonal anti-SSTR2 rabbit antibody (1:200, cat. no. 3582-1; Epitomics, Burlingame, CA) by immunohistochemical analysis as described by Taniyama et al. (23), and by immunofluorescence double labeling with mouse monoclonal antiglucagon (GeneTex, San Antonio, TX) and anti-insulin antibodies (Sigma-Aldrich, St. Louis, MO), as we described previously (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 a two-tailed unpaired Student t test or ANOVA followed by a Tukey honestly significant difference post hoc test (Graphpad Prism Software, San Diego, CA), adjusting for multiple comparison when necessary. Significance was assigned when $P < 0.05$.

RESULTS

Hypoglycemic clamp experiments

Glucose and insulin requirements. As mentioned, the controls used in this study were nondiabetic BBDP rats

that, because of islet inflammation (insulinitis), may be considered to be 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. 1*A*; −60 min), i.e., before insulin, glucose, or SSTR2a infusion, blood glucose levels were not significantly different between the four groups (nondiabetic vehicle: 7.84 ± 0.27 mmol/L; nondiabetic SSTR2a: 8.05 ± 0.51 mmol/L; diabetic vehicle: 7.19 ± 0.43 mmol/L; diabetic SSTR2a: 7.43 ± 0.43 mmol/L). At 0 min, the nondiabetic SSTR2a group (7.55 ± 0.30 mmol/L) also was not significantly different from the nondiabetic vehicle group (6.97 ± 0.25 mmol/L). This also was true for the diabetic rats (vehicle: 6.41 ± 0.32; SSTR2a: 6.92 ± 0.58 mmol/L). The diabetic rats had

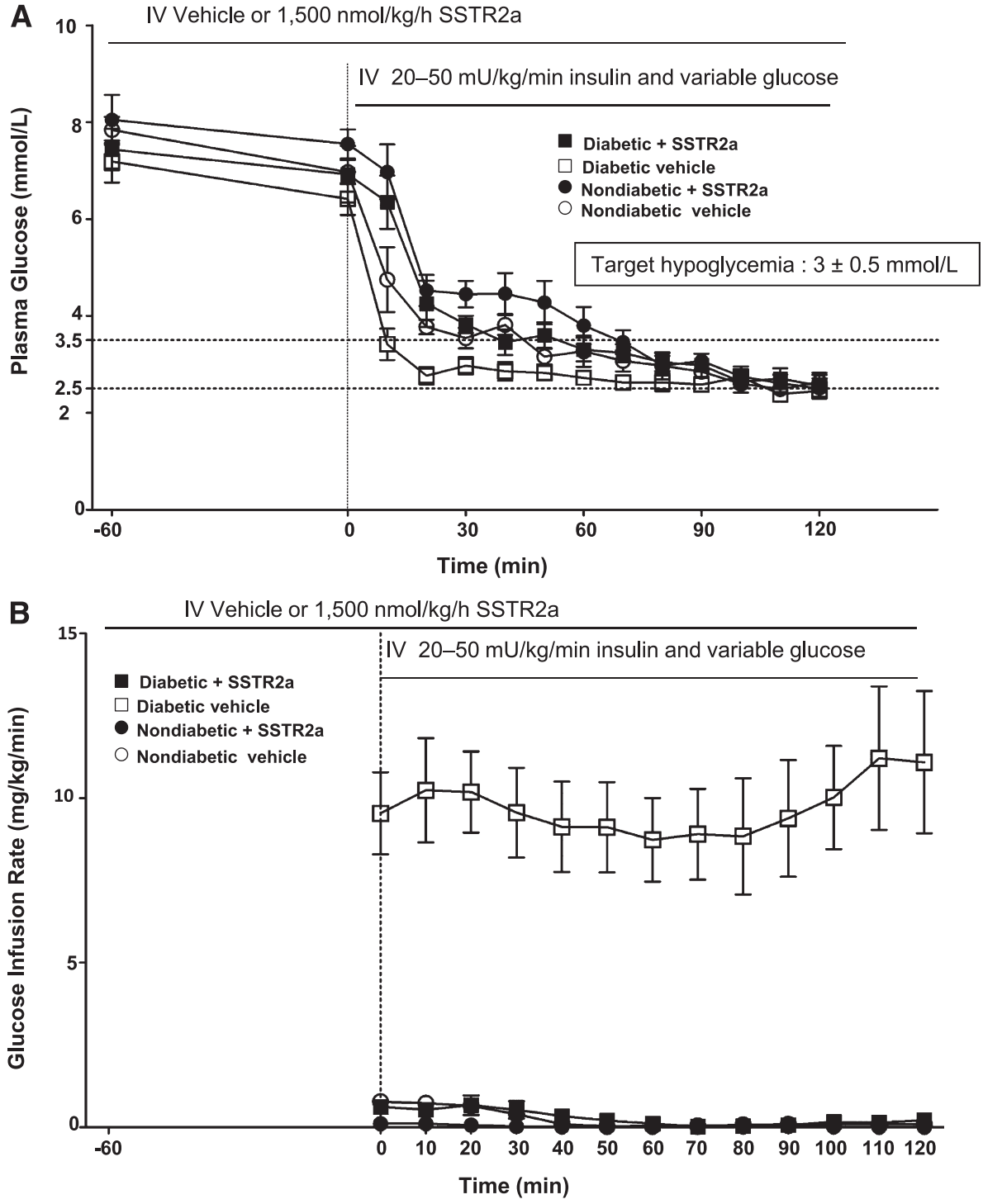

FIG. 1. ***A*: Plasma glucose levels during hyperinsulinemic hypoglycemic clamp experiments (vehicle or 1,500 nmol/kg/h SSTR2a and 20–50 mU/kg/min insulin).** ***B*: Glucose infusion rates during hyperinsulinemic hypoglycemic clamp experiments. ■, *n* = 8; □, *n* = 8; ●, *n* = 6; ○, *n* = 5. Data are presented as means ± SEM. IV, intravenous.**

generally lower glucose levels because 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 min after the start of the insulin infusion and maintained at this level by the variable glucose infusion until the end of the experiment (Fig. 1*A*). To maintain this glucose level, the diabetic vehicle group required very high glucose infusion (area under the curve [AUC]: 1,157 ± 146.6), as would be expected based on the poor counterregulation to hypoglycemia (Fig. 1*B*). In contrast, all other groups required very low or no glucose infusion (all AUCs <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 nondiabetic vehicle (26 ± 2.4 4 mU/kg/min) groups; however, the insulin requirement for the nondiabetic SSTR2a group was higher, at 50 mU/kg/min, to maintain the target hypoglycemic level. This could reflect the fact that nondiabetic BB rats were more insulin-resistant during hypoglycemia (19), perhaps because of their augmented glucagon response to SSTRa treatment (see DISCUSSION).

Effects on hormone secretion: glucagon, corticosterone, catecholamine release, and SST. In the diabetic vehicle group, glucagon secretion during hypoglycemia was low (AUC: 8,982 ± 715; Fig. 2*A*) compared with that of the nondiabetic vehicle group (AUC: 20,679 ± 2,201). With SSTR2a treatment, the diabetic SSTR2a group showed an increase in glucagon secretion (AUC: 21,722 ± 2,962), which was 2.4-fold that of the diabetic vehicle group, indicating full restoration in glucagon secretion. SSTR2a treatment also enhanced the glucagon secretion in the nondiabetic SSTR2a group (AUC: 69,897 ±4,806) to ~3.3-fold compared with the nondiabetic vehicle group.

For corticosterone secretion (Fig. 2*B*), the diabetic vehicle group (AUC: 16,025 ± 924.3) was not significantly different from the nondiabetic vehicle group (12,062 ± 507.6). When treated with SSTR2a, the diabetic SSTR2a group showed an increase in corticosterone secretion (22,341 ± 2,195), but SSTR2a did not affect corticosterone secretion in the nondiabetic SSTR2a group (13,255 ± 1,186). Epinephrine and norepinephrine responses to hypoglycemia (Fig. 2*C*) were not significantly different in the four groups.

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

Pancreatic glucagon and SST protein content. We assessed the pancreatic glucagon and SST content in nondiabetic and diabetic BBDP rats at baseline (without or with treatment with insulin pellets to prevent hypoglycemia) (Fig. 3*A*), and in those that were subjected to the hypoglycemic clamps (Fig. 3*B*).

No hypoglycemic clamp. Pancreatic glucagon (Fig. 3*A*, left panel) and SST (Fig. 3*A*, right panel) protein content in insulin pellet–treated diabetic BBDP rats were both lower than in untreated diabetic and nondiabetic BBDP rats. This would suggest that insulin treatment to induce more normoglycemic control would reduce the demand on α-cells and D-cells to synthesize more glucagon and SST, respectively. This could reflect the absence of endogenous insulin.

After hypoglycemic clamp. Pancreases resected from nondiabetic and insulin pellet–treated diabetic BBDP rats after the insulin-induced hypoglycemic clamp (Fig. 3*B*, left panel) showed that whole pancreatic glucagon protein content was lower in the diabetic SSTR2a group (11.94 ± 0.67 ng/mg protein) compared with the diabetic vehicle group (18.28 ± 1.36 ng/mg protein; $P < 0.001$), and tended to be lower compared with the nondiabetic 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 SST content (Fig. 3*B*, right panel) in the diabetic groups (vehicle: 64.65 ± 5.21 pg/mg protein; SSTR2a: 52.11 ± 2.89 pg/mg protein). However, the SST content in the nondiabetic group (81.95 ± 8.86 pg/mg protein) was increased by SSTR2a treatment (nondiabetic SSTR2a: 118.7 ± 13.27 pg/mg protein) (Fig. 2*D*). At the present time, we cannot clarify why pancreatic SST content immediately after the clamp was elevated in the nondiabetic SSTR2a group as compared with the other three groups.

BBDP rat pancreatic slice perifusion. We first assessed the SSTR2 distribution in the islets of BBDP rat pancreatic sections (Fig. 4*A*). In nondiabetic BBDP rat islets, SSTR2-containing α-cells were distributed more toward the islet periphery. In the shrunken diabetic BBDP islets resulting from reduced β-cell mass, SSTR2-containing α-cells were redistributed more to the islet core. SSTR2 was present only in glucagon-staining α-cells and not in insulin-staining β-cells (few β-cells remaining in diabetic BB rat islet). In Fig. 4*B*, 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 administered together with the antagonist greatly increased glucagon secretion rate. These in vitro results indicate that the increased blood levels of glucagon observed in Fig. 2*A* in both diabetic and nondiabetic BBDP rats were largely attributed to the direct effects of SSTR2a on the pancreatic α-cells.

Human pancreatic slices and islet perifusion. We next assessed whether our finding in BBDP rats applied to human pancreatic α-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, for which we performed immunohistochemistry (Fig. 5*A*) and pancreas slice perfusion assay (Fig. 5*B*) to simulate the results obtained from the BBDP rats. We also used the conventional human isolated islets and subjected them to islet perifusion assay (Fig. 5*C*). A recent report demonstrated that SSTR2 are in human α-cells and β-cells (24). Thus, we first assessed the distribution of SSTR2 in the human pancreas section and found SSTR2 to be in both glucagon-staining α-cells and insulin-staining β-cells (Fig. 5*A*),

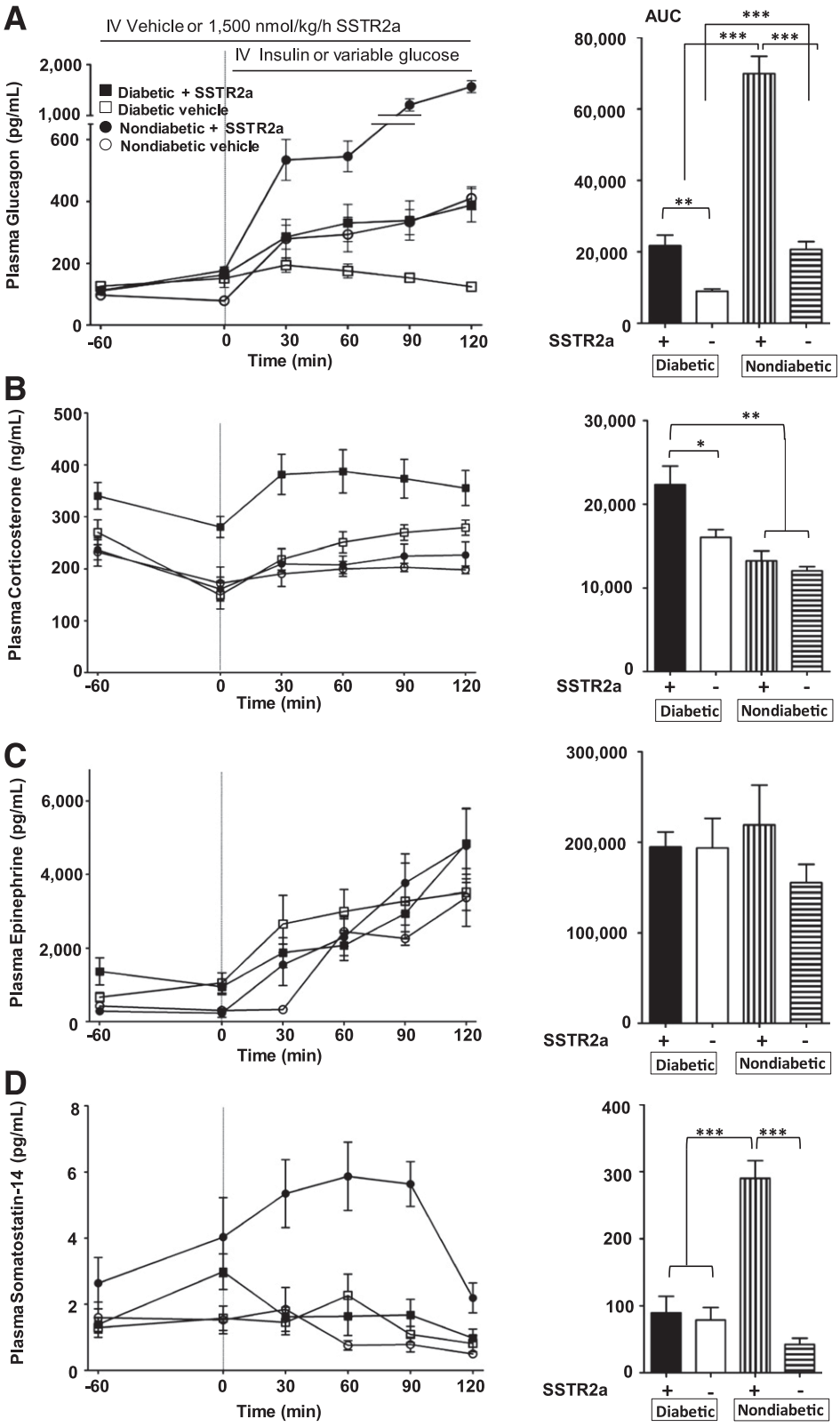

FIG. 2. Plasma hormone levels during hyperinsulinemic hypoglycemic clamp experiments (vehicle or 1,500 nmol/kg/h SSTR2a and 20–50 mU/kg/min insulin) and corresponding AUCs. *A*: Effect of SSTR2a on glucagon response. *B*: Effect of SSTR2a on corticosterone response. *C*: Effect of SSTR2a on epinephrine response. *D*: Plasma SST levels. ■, $n = 8$; □, $n = 8$; ●, $n = 6$; ○, $n = 5$. AUCs were calculated using Prism software (GraphPad Software, San Diego, CA). Filled bars, $n = 8$; open bars, $n = 8$; vertically striped bars, $n = 6$; horizontally striped bars, $n = 5$. Data are presented as means ± SEM. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$. IV, intravenous.

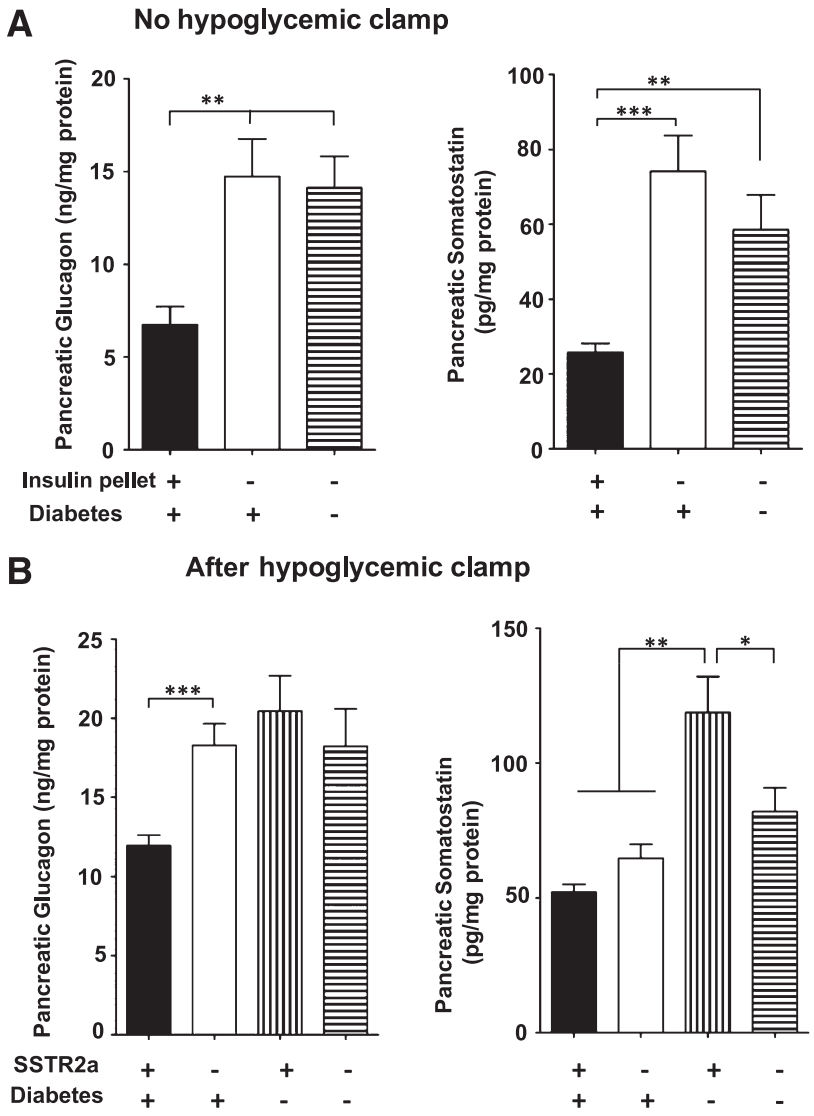

FIG. 3. Whole pancreatic protein contents of glucagon and SST. *A*: Nondiabetic and diabetic BBDP rats (with and without insulin pellet implants) that have not undergone clamp experiments. Filled bars, *n* = 6; open bars, *n* = 6; horizontally striped bars, *n* = 5. *B*: Pancreases were obtained immediately after the hyperinsulinemic hypoglycemic clamp experiments. Diabetic BBDP rats had been treated with insulin pellet implants before the clamp experiments. Filled bars, *n* = 8; open bars, *n* = 8; vertically striped bars, *n* = 6; horizontally striped bars, *n* = 5. Data are presented as means ± SEM. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

and both cell types were mixed within the islet, including the islet core. Thus, we examined whether SSTR2a could influence both glucagon and insulin secretion.

We show two experiments for human pancreas slices (Fig. 5*B*); 1 mmol/L glucose- stimulated glucagon secretion was enhanced by the addition of SSTR2a in both experiments (top, Fig. 5*B*). It was difficult to obtain pancreas specimens from pancreatic cancer resections; therefore, we also used conventional human islets (Fig. 5*C*). We observed similar results of SSTR2 potentiation of glucagon secretion (AUCs: 1 mmol/L glucose plus SSTR2a, 9.53 ± 0.77; 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. 5*B*) or isolated islets (not shown). Addition of SSTR2a had no significant effect (isolated islet AUCs: 1 mmol/L glucose plus SSTR2a, 11.65 ± 2.21; 1 mmol/L glucose, 13.19 ± 1.79; $N = 4$; not significant). This is likely because 1 mmol/L glucose was nonstimulatory on β-cells and because stimulatory glucose concentrations are required for SSTR2a to potentiate insulin release (12). Arginine (Fig. 5*B*) evoked release of more glucagon (at stimulatory glucose) and insulin (at inhibitory glucose), which serve as a positive control and an indicator that the slices were healthy.

DISCUSSION

Previously, in rats with STZ-induced diabetes, we showed that antagonizing SSTR2 fully restores the glucagon and corticosterone counterregulation to insulin-induced hypoglycemia (10). The STZ model is not a genuine disease model for humans with T1D as 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 T1D (2). In the current study, we

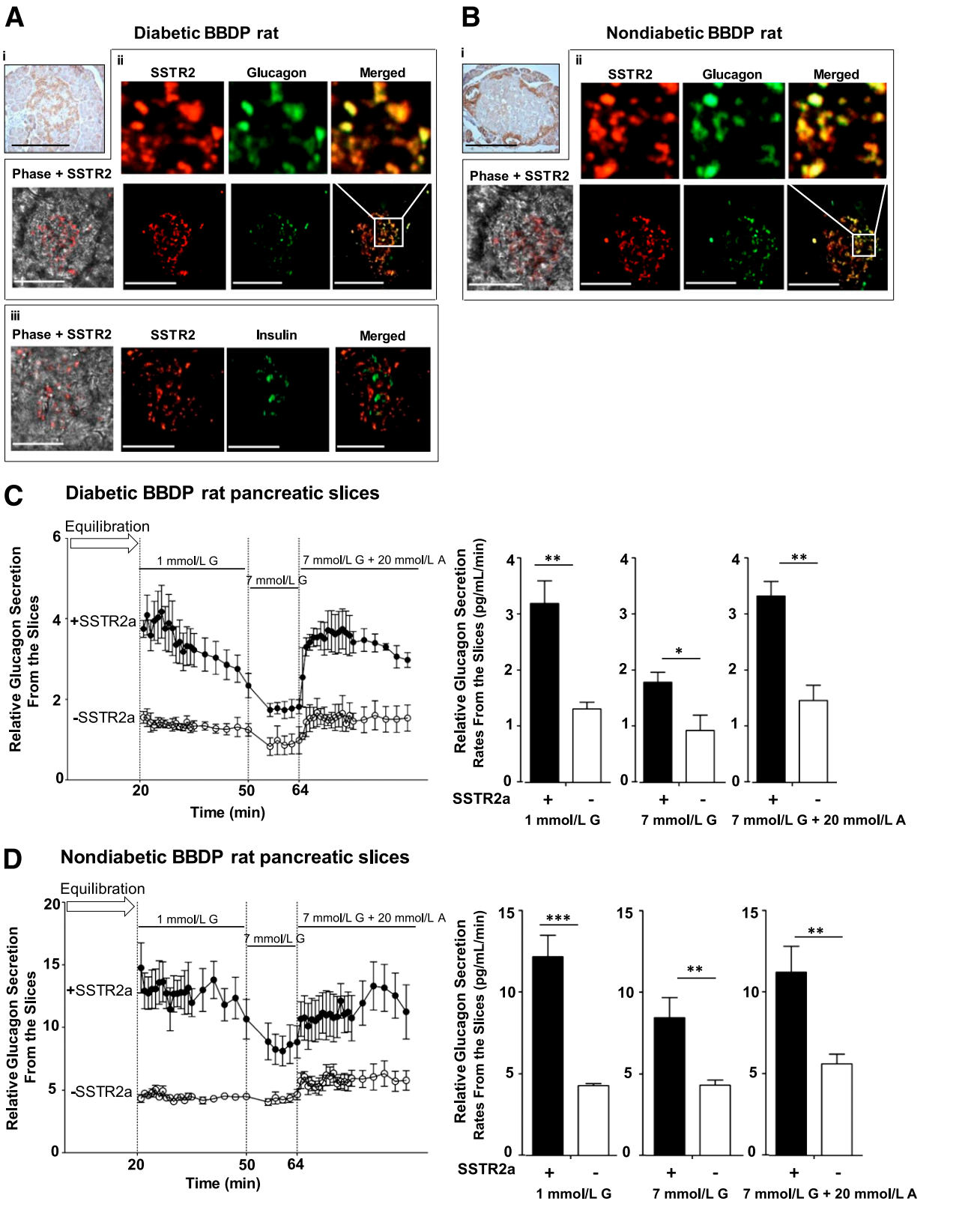

FIG. 4. Effects of SSTR2a on glucagon secretion from pancreatic slices of BBDP diabetic and nondiabetic rats. Immunohistochemistry (brown, SSTR2 labeling [*i*]) and immunofluorescence (red, SSTR2; green, glucagon [*ii*] or insulin [*iii*]; merged images) of the localization of SSTR2 to islet α-cells in BBDP diabetic (*A*) and nondiabetic (*B*) rat pancreas slices. Insets in *ii* are enlarged views showing SSTR2 and glucagon colocalization. In *Aiii*, the few surviving β-cells in diabetic BBDP rats do not contain SSTR2, as is also true in nondiabetic rats (data not shown). Scale bar, 100 μm. Glucagon secretion from pancreatic slices of diabetic BBDP rats ($n = 6$) (*C*) and nondiabetic BBDP rats ($n = 6$) (*D*). Relative glucagon secretion was calculated as the glucagon secretion from the slices normalized 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. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. A, arginine; G, glucose.

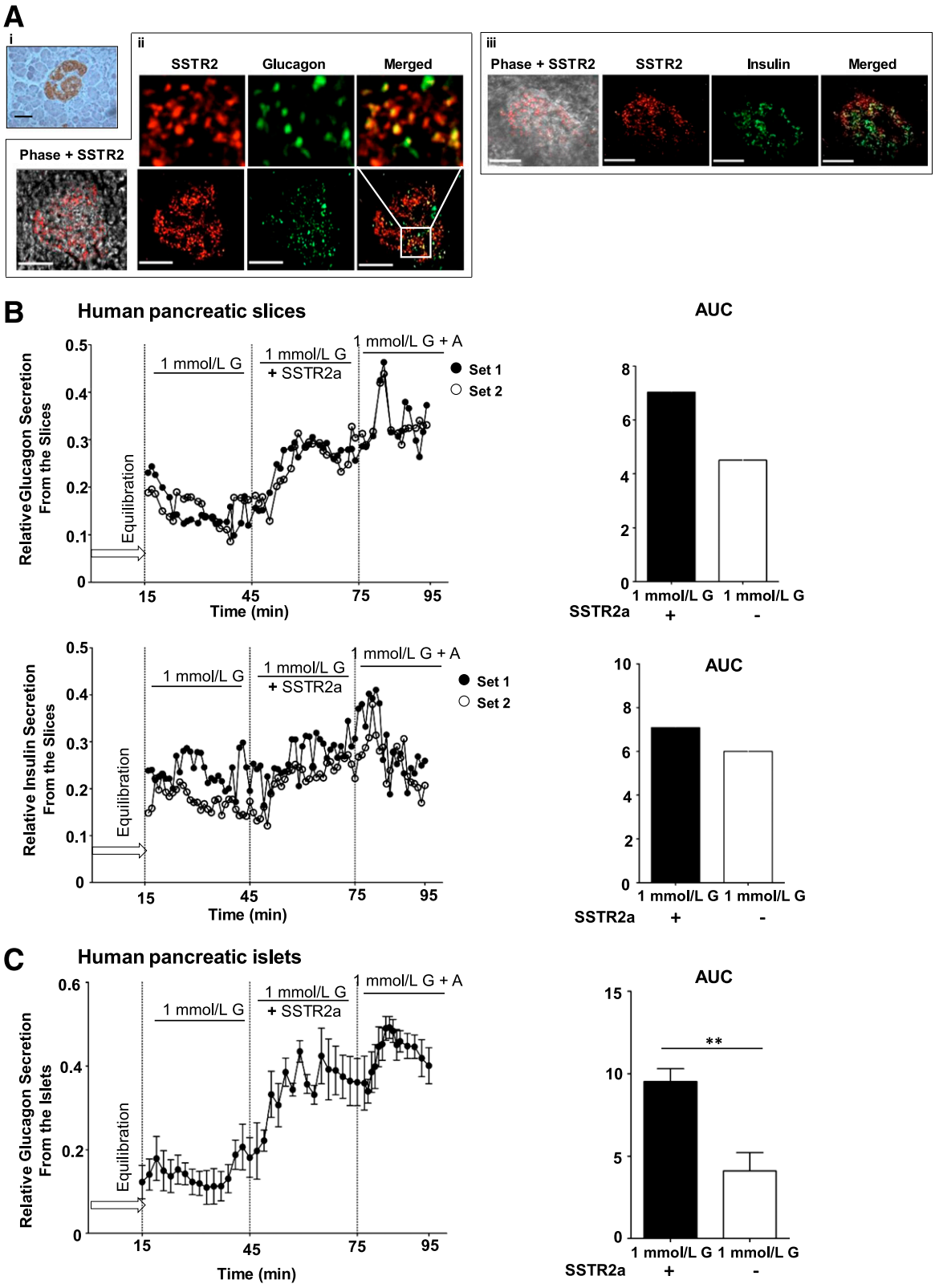

FIG. 5. Effects of SSTR2a on glucagon and insulin secretion from normal human pancreatic slices and isolated islets. *A*: Immunohistochemistry (brown, SSTR2 labeling [*i*]) and immunofluorescence (red, SSTR2; green, glucagon [*ii*] or insulin [*iii*]; merged images) of the localization of SSTR2 to islet α-cells (*ii*) and β-cells (*iii*) in normal human pancreas slices. Inset in *ii* is an enlarged view showing SSTR2 and glucagon colocalization. Scale bar, 100 μm. *B*: Relative glucagon (*top*) and insulin (*bottom*) secretion from pancreatic slices. The filled circles and empty circles represent two sets of perifusion. *C*: Relative glucagon secretion from isolated pancreatic islets ($n = 4$). Relative glucagon or insulin secretion was calculated as the glucagon/insulin secretion from the slices/islets normalized 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. **$P < 0.01$. A, arginine; G, glucose.

demonstrate for the first time that antagonizing the SSTR2 bound to α-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 a major impact on improving glucose counterregulation in diabetic rats, because SSTR2a-treated animals did not require glucose infusion during the hypoglycemic clamp but the untreated diabetic BBDP rats required large amounts of glucose infusion.

Our controls were nondiabetic BBDP rats, which have a low-grade inflammation in the islets before destruction of β-cells, causing rapid progression to severe diabetes requiring exogenous insulin to survive (18,19), thus mimicking the developing pathology in human T1D. Therefore, we 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 nondiabetic BBDP rats enhances glucagon secretion during insulin-induced hypoglycemia. However, the previous study of normal Sprague-Dawley rats (10) did not show any enhancement of glucagon secretion by SSTR2a treatment. This could reflect the fact that nondiabetic BBDP rats are actually in a prediabetic stage of the disease. In fact, when nondiabetic BBDP rats were challenged with experimental hypoglycemia, they showed glucose intolerance that was attributed to reduced insulin release from the islets (19). Therefore, we hypothesize that in addition to the effects of exogenous insulin, inflammatory mediators released during prediabetic insulinitis (18) may have sensitized α-cells to SST 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. However, we did 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 T1D.

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

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

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 (nondiabetic 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 also can 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 T1D autoimmune injury and scarring are considerably smaller in size and have a distorted shape and architecture. Any attempt to isolate them through exposure to enzymatic digestion will damage them (22). Furthermore, in contrast to human islets, α-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 slice perfusion, we observed that SSTR2a significantly enhances the glucagon response during hypoglycemia in diabetic and nondiabetic BBDP rats. This finding further confirms the paracrine inhibitory effect of pancreatic SST on glucagon secretion, which is mediated via the SSTR2 on α-cells.

We also observed that the basal glucagon secretion (at 7 mmol/L glucose) is moderately enhanced in diabetic and nondiabetic BBDP rats. A recent model was postulated to explain the basal hyperglucagonemia in T1D, which is that glucagon secretion has two components (28). The first is autofeedback 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 T1D, it is important to find out whether SSTR increases basal or postprandial glucagon secretion, the latter of which may be driven by an increase in amino acid levels. Our previous study of STZ diabetic rats indicated that SSTR2a infused for 4 h 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. Using the 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 serve to validate the human pancreas slice perifusion assay. This model is ideal for future studies of 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,

because stimulatory glucose concentrations are required for SSTR2a-potentiated insulin release (12). It was shown that SSTR2 is the functionally predominant SST receptor on α-cells and β-cells in human pancreas (12,24). Our findings, taken together with those of other studies, suggest that SSTR2a can improve glucagon secretion in T1D with only α-cells present, and that it also may improve insulin secretion when there are still some residual β-cells.

The potent effects of the SSTR2a on normal human pancreas may be attributable to the fact that the population of D-cells in human islets is approximately twice that of rodent islets, whereas β-cells populations are smaller in human islets than in rodent islets (27). This would suggest that the proportionate inhibitory effects of insulin and SST on human α-cells may be different from those on rodent α-cells, with SST probably having a greater effect on the former. Because SSTR2a can restore glucagon secretion in BBDP rats, preventing insulin-induced hypoglycemia, there could be important clinical implications in using SSTRas to prevent iatrogenic hypoglycemia in type 1 diabetic patients with tight glycemic control (1).

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N.K. performed most of the experiments, contributed to their design and data analysis, and wrote the manuscript. T.Q. performed most of the experiments and contributed to their design and data analysis. T.L., M.O., Y.H., and T.T. contributed to some experiments and to the discussion. M.C.R., M.S.C., and D.H.C. contributed reagents and to the discussion. M.V. and H.Y.G. wrote the manuscript. H.Y.G. is the guarantor of this work and, as such, had 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.

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