Amelioration of Hypoglycemia via Somatostatin Receptor Type 2 Antagonism in Recurrently Hypoglycemic Diabetic Rats

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Abstract

Selective antagonism of somatostatin receptor type 2 (SSTR2-) normalizes glucagon and corticosterone responses to hypoglycemic clamp in diabetic rats. The purpose of this study is to determine if SSTR2 antagonism (SSTR2a) ameliorates hypoglycemia in response to overinsulinization in diabetic rats previously exposed to recurrent hypoglycemia. Streptozotocin-diabetic rats (n= 19), previously subjected to 5 hypoglycemia events over 3 days, received an i.v. insulin bolus (10 U/kg) + insulin infusion (50 mU/kg/min) until hypoglycemia ensued (≤ 3.9 mM) (Expt-D1). The next day (Expt-D2), rats were allocated to receive either placebo treatment (n=7) or SSTR2a infusion (3000 nmol/kg/min, n=12) 60-min prior to same insulin regimen. On Expt-D1, all rats developed hypoglycemia by ~90 minutes, while on Expt-D2 hypoglycemia was attenuated with SSTR2a treatment (nadir = 3.7 ± 0.3 mM vs. 2.7±0.3 mM in SSTR2a and controls, p<0.01). Glucagon response to hypoglycemia on Expt-D2 deteriorated by 20-fold in the placebo group (P<0.001) but improved in the SSTR2a group (3-fold increase in AUC, P<0.001). Corticosterone response deteriorated in the placebo treated rats on Expt-D2 but increased 2-fold in the SSTR2a group. Catecholamine responses were not affected by antagonism. Thus, SSTR2 antagonism following recurrent hypoglycemia improves the glucagon and corticosterone responses and largely ameliorates insulin-induced hypoglycemia in diabetic rats.
Introduction

The management of type 1 diabetes is impeded by the constant threat of hypoglycemia, caused by the inability to achieve physiological insulin replacement and because of a failure in the hormone counterregulation to hypoglycemia (1). Recurrent hypoglycemia increases the susceptibility to subsequent hypoglycemia, since it contributes to both defective hormone counterregulation and to reduced symptom recognition (2). The reduction in symptom recognition for hypoglycemia has profound impact on patient quality of life, and this population fears hypoglycemia more than long-term complications (3, 4). The elevated risk of recurrent hypoglycemia, often precipitated by intensive insulin therapy, frequently necessitates a “relaxation” in management, which ultimately places the individual at risk for earlier complications (3). Currently, there are few prophylactic strategies that limit the risk of developing insulin-induced hypoglycemia (5), perhaps because the neuroendocrine mechanism(s) of impairment have yet to be fully elucidated. None of these treatments would be considered a preventative pharmacological approach.

With repeated exposure to hypoglycemia, there are impairments in the neuroendocrine and autonomic responses to subsequent hypoglycemia (6-9), perhaps because of defects in the regions of the central nervous system that detect and respond to hypoglycemia (1). In addition to numerous neuroendocrine deficiencies related to glucose sensing and blunted counterregulatory responses because of central deficiencies (7, 10-14), elevation in circulating somatostatin levels in type 1 diabetes
has long been thought to impair the counterregulatory response to insulin-induced hypoglycemia (15-20).

Somatostatin acts on various receptor subtypes (SSTR1-5), being both a regulator of hormone secretion (typically inhibitory) and a neurotransmitter (21). With respect to glucose counterregulatory hormones, somatostatin release in the brain lowers pituitary growth hormone secretion indirectly via hypothalamic suppression of GHRH release and directly by acting on somatotrophs via SSTR2 and 5 (22). In the adrenal gland, somatostatin inhibits acetylcholine stimulated medullary catecholamine secretion and inhibits corticosteroid secretion predominantly via SSTR2 (23). In humans, somatostatin lowers pancreatic glucagon and insulin release through SSTR2 (24). In rats, somatostatin inhibits insulin secretion predominantly through SSTR5 (25) and glucagon secretion exclusively through SSTR2 (21).

Paradoxically, somatostatin concentrations are elevated at baseline and rise further during hypoglycemia in patients with type 1 diabetes who are on exogenous insulin (19). Various animal models of type 1 diabetes (7, 17, 18, 26), and isolated islet studies in healthy rats (27), have demonstrated that elevations in somatostatin limit the glucagon response to hypoglycemia and/or arginine stimulation via SSTR2 activation. Since somatostatin also inhibits the release of all of the key hormones involved in glucose counterregulation (i.e. cortisol, growth hormone, catecholamines) (21, 28), an elevation in somatostatin levels in type 1 diabetes may be one of the reasons why glucose counterregulation fails. Accordingly, the systemic administration of a somatostatin receptor agonist exacerbates severe hypoglycemia in patients with type 1 diabetes (29), likely because of reductions in glucose counterregulatory hormone levels.
to ensuing insulin-induced hypoglycemia. Thus, the use of a selective somatostatin receptor 2 antagonist (SSTR2a) may be helpful in improving glucose counterregulation in this patient population. In support of this, we recently demonstrated that SSTR2a (PRL-2903) normalizes the glucagon and corticosterone responses to hypoglycemic clamp in diabetic rats (26). Since these were glucose clamp experiments, it was not possible to determine whether hypoglycemia could be prevented with SSTR2 antagonism. It is also unclear if the improvement in the counterregulatory hormone response caused by SSTR2a would have favourable effects on glucoregulation in diabetes. In this present work, we tested the hypothesis that hypoglycemia can be prevented/attenuated with SSTR2 antagonism treatment in animals previously exposed to repeated hypoglycemic challenge by enhancing counterregulatory responses. We demonstrate here that the glucagon and corticosterone responses improve by SSTR2 antagonism, and that the depth and duration of hypoglycemia is ameliorated in diabetic rats previously exposed to recurrent hypoglycemia.

**Methods**

*Design and experimental animals.* This was a repeated measures randomized design study (placebo/placebo vs. placebo/SSTR2a) to test the effectiveness of SSTR2 antagonism on glucose and hormonal counterregulation during insulin-induced hypoglycemia in diabetic rats previously exposed to recurrent hypoglycemia. Nineteen male Sprague-Dawley rats (Charles River Laboratories, Saint-Constant, QC, Canada) with an initial body mass of 275-300 g were used. Rats were individually housed in opaque cages in a light- and temperature-controlled environment (12-h light:12-h dark
cycle, 20-22°C) and fed ad libitum with chow (Harlan Laboratories, Madison, WI) with free access to food and water. After 1 week of experimenter handling and acclimatization, rats were given a single intraperitoneal streptozotocin (STZ) injection (65 mg/kg dissolved in 0.9% saline; Sigma) to induce diabetes. STZ-injected rats that did not become hyperglycemic within 48 h were excluded from the study. Morning (fed) glycemia (Ascencia Elite handheld glucometer, Bayer Canada Ltd., Etobicoke, ON, Canada), body mass, and food intake were measured daily. Using aseptic technique, rats were anesthetised and catheterized with indwelling cannulae in the left carotid artery and right jugular vein 14 days after STZ injection. The cannulae were exteriorized, fed through a metal coil tether, and connected to a swivel system (rodent tether and swivel; Lomir Biomedical Inc., Notre-Dame-de-l’Île Perrot, QC, Canada). This rodent tethering system allowed for manual, undisturbed blood sampling and infusions (arterial and venous catheters, respectively) and unrestricted movement of the rat while protecting the catheters. Catheters were flushed daily with heparinized (10 USP U/mL) saline to ensure patency. Eighteen days following STZ injection, rats were subjected to recurrent hypoglycemia treatment over 3 days via a hyperinsulinemic/hypoglycemic clamp technique (see below for details). Twenty-one days following STZ injection, rats underwent a standardized 2-day back-to-back hypoglycemic challenge via insulin infusion either with or without SSTR2a (see below for details). All procedures were in accordance with Canadian Council on Animal Care Standards and were approved by the Animal Care Committee of the University of Toronto.
Recurrent hypoglycemia treatment. Nineteen (n=19) rats were subjected to 5 episodes of recurrent hypoglycemia over 3 days using a modified hyperinsulinemia-hypoglycemic clamp technique. Rats were partially fasted overnight (10-15 g of rat chow or ~25-40% of ad libitum consumption with free access to 5% sucrose) prior to each day of recurrent hypoglycemia. On each morning of hypoglycemic challenge, basal blood glucose was measured at t=0 min, and insulin (10 U/kg) was injected subcutaneously to induce hypoglycemia. Glucose infusions (50% dextrose) were given at a variable rate to clamp glycemia at a target hypoglycemia of 3.0±0.5 mM. Blood glucose was measured (Analox glucose analyzer, GMD-9D, Analox Instruments USA Inc., Lunenburg, MA) in duplicate every 15 min for 180 min. During a rest period between 180 and 240 min, rats were given access to 5% sucrose water and intravenous glucose infusion to recover. At 240 min, rats again underwent hypoglycemic challenge until 420 min. Food and sucrose water were fed to aid recovery after hypoglycemia treatment.

Experimental Days 1 and 2. Following this hypoglycemic conditioning period, each rat then underwent 2 additional “experimental days” of hypoglycemic challenge (i.e. with or without SSTR2a treatment), with measurements of their hormonal and glycemic responses. Rats were partially fasted overnight prior to each experimental day, as described above, to allow for standardization in food intake and preservation of liver glycogen stores. In the morning, rats were weighed, connected to venous infusion lines, and acclimated for 2 h prior to experimentation. Basal blood samples for glucose and hormones were taken at the start of the experiment (t=-60 min) from freely moving, conscious rats with cannulas exteriorized outside of the cage. On Experimental Day 1 (Expt-D1), which served as the “control” day to measure the extent of counterregulatory
failure caused by recurrent hypoglycemia, 0.9% saline infusion (1 mL/h) was started in all rats (n=19) after basal samples were obtained at t=-60 min. Blood glucose levels were measured in duplicate using a glucose analyzer at times -60, -40, -20, 0, and every 10 min thereafter until 180 min. Blood samples for glucagon, catecholamines, and insulin were collected in chilled tubes containing EDTA (Sangon Ltd. Canada, Scarborough, ON, Canada) and Trasylol (Bayer Canada Ltd., Etobicoke, ON, Canada). Blood samples for corticosterone were collected in chilled tubes containing heparin. After plasma was removed, packed red blood cells were re-suspended in heparinized saline (10 USP U/mL) containing 1% bovine serum albumin and re-infused into the rat. After blood samples were obtained at t=0, an intravenous insulin bolus (10 U/kg) was administered. To achieve hypoglycemia with as little insulin administered as possible, an intravenous insulin infusion (50 mU/kg/min) was commenced and terminated at the experimenter’s discretion. Infusions were delivered via digital pumps (Harvard Apparatus PHD 22/2000 syringe pumps, Holliston, MA), and both the volume of insulin infused and time when infusion was stopped were recorded. The purpose of Expt-D1 was to attempt to determine the minimal amount of insulin necessary to induce hypoglycemia (2.0-3.5 mM) without causing coma or convulsions and to serve as the control for glucose levels and hormonal responses for Expt-D2. Determining the insulin dosage specifically for each rat on Expt-D1 was necessary since insulin sensitivities of these diabetic rats varied. It is worth noting that neither glucose infusions nor SSTR2a were given on Expt-D1 since it was important to examine each animal’s capacity to counterregulate following recurrent hypoglycemia. On Expt-D2, rats were randomly allocated to SSTR2a (n=12) or placebo (n=7) treatment. A greater number of rats were
given the SSTR2a since it was expected that results would be more variable in this
group. The insulin regime on Expt-D2 was identical to that used on Expt-D1 for a given
rat so that any differences in treatment (placebo vs. SSTR2a) could be observed.
Infusion of SSTR2a (PRL-2903, 3000 nmol/kg/min at 1 mL/h) was commenced at t=-60
min and continued for 5-h duration of the experiment, as previously described (26), to
determine the effect of SSTR2 antagonism on the depth and duration of hypoglycemia.
In the placebo-treated group, saline was infused in place of SSTR2a. At the end of 240
min on Expt-D2, all rats were quickly euthanized by decapitation.

**Plasma hormone measurements.** Plasma glucagon and insulin (LINCO Research Inc.,
St. Charles, MO), catecholamines (LDN GmbH & Co. KG; Nordhorn, Germany), and
corticosterone (MP Biomedicals, Solon, OH) were measured using radioimmunoassay
using commercially purchased kits as previously described (26).

**Somatostatin receptor type 2 antagonist.** This peptide antagonist (PRL-2903, BIM-
23458) was synthesized and provided by Dr. David Coy (Tulane University, New
Orleans, LA). Solutions of the peptide antagonist dissolved in 1% acetic acid and
diluted with 0.9% saline were freshly prepared the morning of the experiment.

**Data analysis.** All data are represented as means ± SEM. Main outcomes were the
repeated measure comparisons of counterregulatory hormones and glycemic responses
between Expt-D1 and Expt-D2 in the two groups of rats (placebo vs. SSTR2a-treated).
Areas under the curve (AUC) were calculated using Prism software (GraphPad
Software, San Diego, CA). Statistical analysis was performed using Statistica software
(Statsoft Inc., Tulsa, OK) on the glycemic responses and the AUCs for
counterregulatory hormone responses. Glucose measurements taken over time were compared using repeated measures ANOVA, followed by Duncan's post-hoc test. Other comparisons between Expt-D1 and Expt-D2 within the same group were assessed using a paired $t$-test while comparisons between groups were conducted via a two-tailed $t$-test. In all tests, significance was deemed when $P<0.05$.

Results

**Daily blood glucose, body weight, and food intake.** In the week prior to recurrent hypoglycemia, fed glucose (23.6±2.1 vs. 25.3±0.9 mM), body mass (372±11 vs. 365±6 g) and food intake (37±2 vs. 39±1 g/day) were similar in rats that would later be divided into the placebo (saline) and SSTR2a-treated groups (all $P>0.05$, respectively).

**Glycemia during recurrent hypoglycemia treatment.** By design, all rats achieved 5 similar episodes of recurrent hypoglycemia over 3 days (nadir= 3.0±0.5 mM for an average of 90 min per episode; Figure 1A).

**Basal blood glucose and plasma hormone levels after recurrent hypoglycemia treatment.** On the morning of Expt-D1 and Expt-D2, body mass and initial glycemia did not differ between groups (Table 1). Circulating basal (i.e. before treatment and hypoglycemia induction) insulin and counterregulatory hormone levels were also similar between groups (Table 2). Thus, all rats had similar metabolic starting points following recurrent hypoglycemia.

**Plasma insulin and blood glucose levels during Expt-D1 and Expt-D2.** As we endeavoured, similar amounts of insulin (bolus and infusion) were administered to both
groups on both days (Table 1). Giving the same amount of insulin on both experimental
days, within a treatment group, was important so that any changes observed with
glycemia would not be attributed to the amount of insulin administered. Peak circulating
insulin levels were also similar between groups and between days (placebo group Expt-
D1: 89.3±32.9, Expt-D2: 71.9±18.7; SSTR2a group Expt-D1: 96.7±15.9, Expt-D2:
75.7±12.3 ng/ml, not significantly different).

In the controls, which received saline infusion on both days, the depth (nadir =
2.6±0.4 vs 2.7±0.3 mM) and duration of hypoglycemia were similar between Expt-D1
and Expt-D2 (Figure 1B). In contrast, in the SSTR2a group, insulin infusion induced a
similar rate of glycemic decline to ~4.0 mM by 90 min on both days, but then blood
glucose levels diverged as the threshold for hypoglycemia was approached (Figure 1C).
More specifically, with SSTR2 antagonism on Expt-D2, both the depth (nadir: 2.9±0.1
vs. 3.7±0.3 mM on Expt-D1 vs. Expt-D2, respectively; P<0.01) and the duration (126±9
vs. 73±13 minutes on Expt-D1 vs. Expt-D2, respectively; P<0.01) of hypoglycemia were
significantly less when compared to Expt-D1 (Figures 1C). In addition, with SSTR2a
treatment, rats remained in the euglycemic range in recovery and did not develop
rebound hyperglycemia, at least for the experimental period examined (up to 240
minutes post insulin administration). The extent of hypoglycemia, calculated as the
AUC <4.0 mM, was considerably less with SSTR2a treatment compared to saline
treatment (10 vs. 90 mM/min, P<0.001). Since some rats still developed hypoglycemia
with SSTR2a treatment, albeit in a milder form, the percent of animals with blood
glucose levels <4.0 mM and <3.5 mM were also plotted for Expt-D2 only, since this was
the only day in which the treatments differed (Figures 2A, 2B). In this analysis of drug
efficacy, the percentage of rats with blood glucose levels <4.0 mM and <3.5 mM were higher in the rats given SSTR2a (~33% and 40%, respectively) compared with rats given placebo (~0% and 8%, respectively).

**Counterregulatory hormone levels during Expt-D1 and Expt-D2.** Since baseline levels of all counterregulatory hormones differed slightly between groups and between days (Table 2), their responses to hypoglycemic treatment were plotted for both groups (Figure 3A-F). Following three days of recurrent hypoglycemia, glucagon responses to hypoglycemia on Expt-D1 were modest in both groups (Figure 3A, 3B). On Expt-D2, the glucagon response to hypoglycemia in controls diminished markedly (AUC decreased by >20-fold, P<0.05), while it improved significantly in the SSTR2a-treated group (AUC increased 3-fold, P<0.05).

In controls, the corticosterone responses to hypoglycemia was relatively robust on Expt-D1, but diminished by approximately half on Expt-D2 (P<0.05, Figure 3C). In contrast, the corticosterone response to hypoglycemia in the SSTR2a group on Expt-D1 was somewhat attenuated compared to controls, but tended to improve with SSTR2a treatment on Expt-D2 (P=0.2 for AUC analysis, Figure 3D).

Epinephrine levels increased in the controls on Expt-D1, but responses were significantly attenuated on Expt-D2 (P<0.05 for AUC, Figure 3E). Interestingly, catecholamine responses were less robust in the SSTR2a group on both experimental days when compared to controls and were still attenuated on Expt-D2 when compared to Expt-D1 (Figure 3F). Since the apparent attenuation in epinephrine response on Expt-D2 compared with Expt-D1 despite SSTR2a treatment may have been related to
improved counterregulation in other hormones and to higher glycemic values overall, we also compared the catecholamine responses between Expt-D1 and 2 in the six rats who still developed hypoglycemia with SSTR2a treatment. In this analysis, we observed that SSTR2 antagonism was associated with a preserved epinephrine response to recurrent hypoglycemia (data not shown). Norepinephrine response to hypoglycemia was similar both between groups and between experimental days (data not shown).

Discussion

This study is the first to show that the use of a selective somatostatin receptor 2 inhibitor reduces the likelihood of insulin-induced hypoglycemia in diabetic rats that have developed counterregulatory failure because of repeated exposure to recurrent hypoglycemia. This novel finding may have significant implications for the development of new prophylactic therapies targeting SSTR2 inhibition for hypoglycemia prevention in type 1 diabetes mellitus.

Our prior work has demonstrated that SSTR2 antagonism improves some of the counterregulatory hormone response to hypoglycemic clamp in diabetic rodents naive to prior hypoglycemia (26). This study extends these findings by showing that rodents exposed to recurrent hypoglycemia have improvements in their glucagon and corticosterone, but not catecholamine, responses to subsequent hypoglycemia when a SSTR2a is administered. More importantly, animals are shown to be more resistant to insulin-induced hypoglycemia with SSTR2a treatment. These findings are particularly relevant since reducing the hypoglycemic nadir and the duration of hypoglycemic exposure are both important in preserving normal brain function and preventing severe
neuroglucopenia, seizures, and loss of consciousness and/or death (3). If selective SSTR2 antagonism is demonstrated to promote “hypoglycemic resistance” in the long-term, without adverse side effects, then insulin therapies that include SSTR2 antagonism may have wider latitude for safety patients living with type 1 diabetes mellitus.

Taken together with our prior study using SSTR2 inhibition (26) and the observations that there are elevations in pancreatic gene expression and somatostatin levels in diabetes (16-20), we propose here that increased somatostatin concentration and/or signalling is one of the key contributing factors in the development of glucose counterregulation failure in type 1 diabetes mellitus. However, this study also reveals that some rodents still develop hypoglycemia even when somatostatin inhibition exists (~50%, Figure 2), perhaps because the glucagon response to hypoglycemia is not fully restored in rats exposed to recurrent hypoglycemia. Indeed, a comparison of glucagon responses in this study with that of non-diabetic rodents and rodents given SSTR2a during their first bout of hypoglycemia in our prior work (26) suggests that the decrement in counterregulatory responses to recurrent hypoglycemia are not fully restored by SSTR2 antagonism (~30 vs. 225 pg/mL glucagon response in this study compared with our prior study). Nonetheless, the ability of the SSTR2a to help preserve blood glucose levels above 3.5 mM in rats previously exposed to frequent hypoglycemia may have clinical relevance as an earlier study demonstrated that hypoglycemia at 3.3 mM reduced cognitive function (30). It remains to be determined, however, if prolonged SSTR2 antagonism therapy can limit the frequency of insulin-induced hypoglycemia in
type 1 diabetes or if it has any efficacy in limiting the high rate of occurrence of hypoglycemia in type 2 diabetes.

Hypoglycemia, even when symptom free, leads to defective glucose counterregulation and hypoglycemia unawareness (31, 32). Since these episodes substantially increase the risk of subsequent hypoglycemia, all hypoglycemic events place the individual at elevated risk for future (and more catastrophic) occurrences (1). It has been suggested that delayed recovery of hypoglycemia may frequently occur in type 1 diabetic individuals in which deficient epinephrine and glucagon counterregulation results in impaired hepatic glucose release (33). In patients with type 1 diabetes, avoidance of hypoglycemia helps to restore hypoglycemia awareness and perhaps glucose counterregulation (5, 34-36). This avoidance is typically done by a “relaxation” in insulin therapy which can cause a deterioration in glycemic control, as measured by hemoglobin A\textsubscript{1C} levels (34-36). Whether SSTR2a treatment deteriorates insulin sensitivity and/or allows for a restoration in glucose counterregulation and hypoglycemia avoidance without any alterations in insulin therapy remains to be established. In this study, it would appear that the insulin pharmacokinetics, as assessed by the rate of change in glucose following i.v. insulin administration, were unchanged with SSTR2a treatment which may be considered beneficial for overall patient control (Figure 1C).

In our prior study, we observed that SSTR2a appeared to promote an increase in glucagon release only during hypoglycemia (26). In this study, it would appear that the SSTR2a may increase glucagon release well before hypoglycemia ensues. Indeed, the
glucagon response in the SSTR2a treated animals rose well before the onset of hypoglycemia (see Figures 1 and 3). This suggests that the antagonist may trigger enhanced hormone release in response to a decline in glycemia rather than to hypoglycemia per se. Although our prior work suggests that the provision of the same SSTR2a does not influence insulin sensitivity or glucose production during euglycemia or hyperglycemia (26), a more generic effect of SSTR2a treatment on glucagon release cannot be ruled out at this time.

There is little doubt that somatostatin levels are increased with diabetes. Prosomatostatin mRNA expression in diabetic rats are elevated in islets when compared to non-diabetic rats, and these levels remain elevated even after 7 episodes of recurrent hypoglycemia (7). In humans with type 1 diabetes, circulating somatostatin levels are elevated (16, 17, 19) and pancreatic somatostatin levels are elevated by more than 10-fold (37, 38), particularly in those with poor glycemic control (38). Thus, as with acute hypoglycemia, we propose here that increased pancreatic somatostatin levels may play a role in impairing glucagon release following recurrent hypoglycemia. Using this same selective SSTR2a, stimulated secretion of glucagon, but not insulin, is dose-dependently enhanced in perifused islets and in perfused pancreata of healthy rats (27). The same antagonist also reverses the suppressive effects of a SSTR2 agonist on arginine-stimulated glucagon secretion in isolated human islets (39). Based on the improved glucagon responses to hypoglycemia that we previously observed with SSTR2a treatment (26) and with our observations in this study (Figure 3B), we assume that the effectiveness of SSTR2 antagonism on hypoglycemia prevention following recurrent hypoglycemia is primarily related to enhanced glucagon-mediated hepatic
glycogenolysis. However, other possible mechanisms do exist. It may be that somatostatin antagonism increases glucose production or lowers glucose disposal. At present, however, there is no known direct effect of SSTR2 antagonism on hepatic glycogenolysis or gluconeogenesis. Prolonged infusion of SSTR2a under basal, non-clamped conditions does not appear to affect glucose turnover (26). Moreover, SSTR1 and SSTR3, but not SSTR2, have been detected on hepatocytes (40, 41) and the SSTR2a used in this study has no reactivity with SSTR1 and 10-fold less binding affinity with SSTR3 (42). As previously observed (26), corticosterone levels tend to be increased during hypoglycemia when SSTR2a is given (Figure 3D). Elevations in corticosterone would also be expected to enhance hepatic glucose production and possibly limit peripheral glucose uptake. Interestingly, a direct effect of somatostatin to enhance insulin-stimulated muscle glucose uptake, but not basal muscle glucose uptake, has been demonstrated in humans (43). Thus, it is plausible that inhibition of somatostatin in the present study could contribute to reduced muscle glucose clearance during hypoglycemia, although overall insulin sensitivity did not appear to be impacted by the use of the antagonist in this study. Evidence of SSTR subtypes on skeletal muscle is scarce, but SSTR2, SSTR3, and SSTR4 mRNA has been detected in rat skeletal muscle (44). Thus, a global reduction in somatostatin signalling may promote several antihypoglycemic mechanisms, some of which remain to be identified.

In addition to attenuated glucagon and corticosterone response to hypoglycemia, the catecholamine responses are also lost (7, 8). Somatostatin is thought to inhibit epinephrine release by receptor-coupled signalling initiated by acetylcholine-nicotinic receptor binding, subsequent membrane depolarization, and intracellular calcium
increase (45), resulting in the consequent inhibition of adrenomedullary epinephrine secretion from the adrenal medulla. However, in our studies, SSTR2a did not improve the catecholamine response to hypoglycemia (Figure 3F). We speculate that the apparent attenuation in epinephrine response to hypoglycemia was because glycemia did not reach the same nadir when SSTR2a was used, likely because the glucagon response was improved. Indeed, in the six rats that developed hypoglycemia despite SSTR2a treatment, the epinephrine response was identical to that observed on Expt-D1. Thus, we conclude that SSTR2a treatment does not significantly alter the catecholamine response to hypoglycemia, at least in rats that had diabetes for a relatively short period of time.

Our study has a number of limitations that should be mentioned. First, in spite of identical hypoglycemic conditioning leading up to the experimental days, the two groups of rats examined had different glucose counterregulatory responses to the hypoglycemia on Expt-D1. Indeed, the corticosterone and epinephrine responses in the rats that would receive SSTR2a the next day appeared to be slightly more impaired when compared to the placebo group (Figure 3), which may have exaggerated the effectiveness of the antagonist on Expt-D2. On the other hand, if this group of rats did have significantly attenuated counterregulatory responses, one may consider that the antagonist is indeed effective even in animals that have completely abolished glucose counterregulation. Second, because of limitations in blood collection, we did not measure the growth hormone response to hypoglycemia in this study, which may have been improved with SSTR2a treatment. In our previous publication (26), we did not observe an effect of SSTR2a inhibition on growth hormone release during hypoglycemia.
(unpublished data). However, given that growth hormone is triggered by hypoglycemia (45) and suppressed by somatostatin primarily via the type 2 receptor (46, 47), further studies are needed to determine if SSTR2a treatment also helps to augment growth hormone release during hypoglycemia.

In conclusion, we demonstrate that hypoglycemia can be ameliorated by SSTR2 antagonism following recurrent hypoglycemia in diabetic rats, presumably at least in part by enhancing glucagon and corticosterone counterregulation. These results also help to support the role for pancreatic, and possibly circulating, somatostatin in attenuating the counterregulatory response in diabetes and that this defect can be countered using a pharmacological dose of SSTR2a. Although further investigation is necessary to elucidate the exact mechanisms by which euglycemia is restored by inhibiting somatostatin action and if any other deleterious off-target effects occur because of regular treatment, these findings hold promise for a new pharmacotherapy for hypoglycemia prevention in type 1 diabetes.
Author contributions: JTYY researched data and wrote the manuscript. MCR assisted with the research data analysis and interpretation and the writing of the manuscript. EB researched data. SE contributed to discussion, reviewed/edited manuscript. DHC provided the antagonist, assisted with the research design and edited the manuscript. MV oversaw the research design and interpretation of the data and reviewed/edited manuscript. MCR is the guarantor of this work and, as such, has full access to all 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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Legend for figures

**Figure 1:** Blood glucose responses to repeated insulin induced hypoglycemia. Antecedent hypoglycemia was induced by subcutaneous insulin injection (10 U/kg) and variable rate glucose infusion over a three day “conditioning” period in all rats (n=19) (Panel A). The next day (Expt-D1), rats were randomly allocated to either the control group (n=7, Panel B) or the SSTR2a treatment group (n=12, Panel C) for baseline assessment of counterregulatory responses, by using a combination of i.v. insulin bolus (10 U/kg), and infusion (50 mU/kg/min) at the discretion of the investigator, until moderate hypoglycemia ensued (target 3.0 mM) (Panel B). One day later, on Expt-D2, the insulin infusion treatment protocol was duplicated for each rat, either with (SSTR2a group) or without (controls) i.v. SSTR2a infusion (3000 nmol/kg/min), which commenced 60 minutes prior to insulin treatment. Values are mean ± SEM.

**Figure 2:** Percentage of rats that developed hypoglycemia, as measured by a blood glucose <4.0 mM (Panel A) or < 3.5 mM (Panel B) in the control and SSTR2a-treated groups on Expt-D2.

**Figure 3:** Counterregulatory hormone responses to hypoglycemia in control and SSTR2a treated rats on Expt-D1 and D2. Plasma glucagon responses (Panels A, B), plasma corticosterone responses (Panels C, D) and plasma epinephrine responses (Panels E, F) were determined for Expt-D1 (open symbols) and Expt-D2 (closed symbols) in both groups. The integrated area under the curve (AUC) for the hormonal responses to hypoglycemia is also shown (insets). Values are mean ± SEM. * denotes Expt-D2 was significantly different from Expt-D1 at P<0.05.
Table 1: Body weight and the amount of insulin administered via iv bolus and iv infusion and total insulin administered in both groups on both days. On Expt-D1, rats received a 10 U/kg iv bolus of insulin after basal samples were obtained at time = 0 min. Subsequently, insulin infusion (50 mU/kg/min) was started and stopped at the experimenter's discretion when the rat's blood glucose approached hypoglycemia. The volume, timing and rate of insulin infusion was recorded and repeated for each individual rat on Expt-D2. No difference existed either between groups or between experimental days.

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<tr>
<td>Baseline glycemia (mM)</td>
<td>20.9 ± 4.0</td>
<td>21.5 ± 4.4</td>
</tr>
<tr>
<td>Insulin via iv bolus (U)</td>
<td>3.12 ± 0.63</td>
<td>3.09 ± 0.64</td>
</tr>
<tr>
<td>Insulin via iv infusion (U)</td>
<td>0.89 ± 0.30</td>
<td>0.89 ± 0.30</td>
</tr>
<tr>
<td>Total insulin administered (U)</td>
<td>4.01 ± 0.86</td>
<td>3.98 ± 0.85</td>
</tr>
</tbody>
</table>
Table 2: Basal plasma hormone levels on the morning of Expt-D1 and D2 in rats that would subsequently undergo hypoglycemia treatment. No significant differences existed either between groups or between days.

<table>
<thead>
<tr>
<th></th>
<th>Control group (saline-saline)</th>
<th>Treatment group (saline-SSTR2a)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Expt-D1</td>
<td>Expt-D2</td>
</tr>
<tr>
<td>Insulin (ng/ml)</td>
<td>1.00 ± 0.2</td>
<td>0.97 ± 0.2</td>
</tr>
<tr>
<td>Glucagon (pg/ml)</td>
<td>55 ± 5</td>
<td>53 ± 5</td>
</tr>
<tr>
<td>Epinephrine (pg/ml)</td>
<td>126 ± 33</td>
<td>129 ± 29</td>
</tr>
<tr>
<td>Norepinephrine (pg/ml)</td>
<td>401 ± 52</td>
<td>378 ± 72</td>
</tr>
<tr>
<td>Corticosterone (ng/ml)</td>
<td>110 ± 37</td>
<td>132 ± 28</td>
</tr>
</tbody>
</table>
References


11. Davis MR, Mellman M, Shamoone H: Further defects in counterregulatory responses induced by recurrent hypoglycemia in IDDM. *Diabetes.* 41:1335-1340, 1992


Blood glucose responses to repeated insulin induced hypoglycemia. Antecedent hypoglycemia was induced by subcutaneous insulin injection (10 U/kg) and variable rate glucose infusion over a three day “conditioning” period in all rats (n=19) (Panel A). The next day (Expt-D1), rats were randomly allocated to either the control group (n=7, Panel B) or the SSTR2a treatment group (n=12, Panel C) for baseline assessment of counterregulatory responses, by using a combination of i.v. insulin bolus (10 U/kg), and infusion (50 mU/kg/min) at the discretion of the investigator, until moderate hypoglycemia ensued (target 3.0 mM) (Panel B). One day later, on Expt-D2, the insulin infusion treatment protocol was duplicated for each rat, either with (SSTR2a group) or without (controls) i.v. SSTR2a infusion (3000 nmol/kg/min), which commenced 60 minutes prior to insulin treatment. Values are mean ± SEM.
Percentage of rats that developed hypoglycemia, as measured by a blood glucose <4.0 mM (Panel A) or < 3.5 mM (Panel B) in the control and SSTR2a-treated groups on Expt-D2.
Counterregulatory hormone responses to hypoglycemia in control and SSTR2a treated rats on Expt-D1 and D2. Plasma glucagon responses (Panels A, B), plasma corticosterone responses (Panels C, D) and plasma epinephrine responses (Panels E, F) were determined for Expt-D1 (open symbols) and Expt-D2 (closed symbols) in both groups. The integrated area under the curve (AUC) for the hormonal responses to hypoglycemia is also shown (insets). Values are mean ± SEM. * denotes Expt-D2 was significantly different from Expt-D1 at P<0.05.