# Somatostatin Receptor Type 2 Antagonism Improves Glucagon and Corticosterone Counterregulatory Responses to Hypoglycemia in Streptozotocin-Induced Diabetic Rats

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Diminished responsiveness to hypoglycemia contributes to defective counterregulation in diabetes. Pancreatic and/or circulating somatostatin are elevated in diabetes, which may inhibit counterregulatory hormone release during hypoglycemia. Thus, a selective somatostatin receptor type 2 antagonist (SSTR2a) should improve hormone counterregulation to hypoglycemia. Nondiabetic (N) and streptozotocin-induced diabetic (D) rats underwent 4-h infusion of saline or SSTR2a with insulin-induced hypoglycemia clamped at 2.5 ± 0.5 mmol/L. To evaluate the effect of the SSTR2a in the absence of hypoglycemia, rats underwent a 4-h infusion of saline (Ctrl:N, Ctrl:D) or SSTR2a (Ctrl:D +SSTR2a) only. The attenuated glucagon response to hypoglycemia in D ($P < 0.0002$) was fully restored by SSTR2a ($P < 0.0001$). Furthermore, the attenuated corticosterone response in D ($P < 0.002$) was also enhanced by SSTR2a ($P < 0.05$). In the absence of hypoglycemia, SSTR2a did not alter basal blood glucose levels. D exhibited 62% more pancreatic somatostatin than N after hypoglycemia. In N rats, SSTR2a did not augment the glucagon or corticosterone response to hypoglycemia. Thus, somatostatin may contribute to impaired glucagon responsiveness to hypoglycemia in diabetes. We demonstrate that SSTR2 antagonism enhances hypoglycemia-stimulated glucagon and corticosterone release in D but not in N rats. SSTR2 antagonism does not affect basal glycemia in D rats. ***Diabetes*** **61:197–207, 2012**

Hypoglycemia is the most serious acute complication of type 1 diabetes and the limiting factor of intensive insulin treatment (1). The problem of hypoglycemia is particularly difficult because counterregulation is impaired in type 1 diabetes. In the normal response to hypoglycemia, insulin secretion decreases, and the release of counterregulatory hormones (glucagon, catecholamines, glucocorticoids, and growth hormone) increases (1). The glucagon response is the first to be defective in diabetes (2,3). The glucagon response can be mediated by central (4,5) and local islet (6,7) factors. It has previously been hypothesized that in type 1 diabetes, α-cells are not exposed to endogenous insulin and become excessively sensitive to exogenous insulin (8). Furthermore, in type 1 diabetes, hypoglycemia cannot decrease endogenous insulin release, which is normally a signal for glucagon release during hypoglycemia (9).

Somatostatin suppresses stimulated α-cell glucagon release, and we suggested 20 years ago that increased somatostatin might be a factor explaining inadequate glucagon release during hypoglycemia in type 1 diabetes (10). Plasma somatostatin and pancreatic prosomatostatin mRNA and somatostatin protein levels are increased in diabetic humans (11), dogs (10), and rodents (12,13), which may be due to insulin deficiency (10,14), although insulin treatment did not prevent the abundance of somatostatin-containing δ-cells in human diabetic pancreata (11), and/or absolute or relative glucagon excess within the pancreatic islet might also lead to a compensatory increase in somatostatin (11). In type 1 diabetes, upper-gut somatostatin, the major source of circulating somatostatin, is also increased (14,15). This led us to wonder whether somatostatin, and especially excessive somatostatin, could be responsible, at least in part, for the defect in glucagon responsiveness to insulin-induced hypoglycemia in diabetes.

The objective of this study was to determine whether the glucagon response to hypoglycemia could be improved or normalized in diabetic rats by removing the suppressive effect of somatostatin on glucagon secretion. In rodent and human islets, somatostatin receptor type 2 (SSTR2) is found nearly exclusively on glucagon-secreting α-cells, whereas SSTR1 and SSTR5 are abundant on human and rodent β-cells, respectively (16,17). Somatostatin receptors (SSTR1–5) are ubiquitously expressed, and SSTR2 is coexpressed with other SSTRs in tissues such as the pancreas, brain, pituitary, kidney, liver, stomach, and lymphocytes (15). In this study, we used an antagonist to SSTR2, by which somatostatin exerts its inhibitory effect on stimulated glucagon secretion. Using this antagonist to SSTR2, Efendic and colleagues (18) demonstrated that arginine-stimulated secretion of glucagon is enhanced in perifused islets and perfused pancreata of nondiabetic rats. We hypothesize that selective SSTR2 antagonism may normalize the glucagon response to hypoglycemia. Intracerebroventricular administration of somatostatin decreases stress-induced glucocorticoid and catecholamine responsiveness (19–21). Therefore, we also evaluated the effect of SSTR2 antagonism on other counterregulatory hormone responses during hypoglycemia. We demonstrate that SSTR2 antagonism restores glucagon and corticosterone counterregulation to hypoglycemia in diabetic rats. In the absence of hypoglycemia,

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this SSTR2 antagonist (SSTR2a) neither elicits hyperglycemia nor markedly elevates counterregulatory hormones in diabetic rats.

## RESEARCH DESIGN AND METHODS

**Experimental animals and design.** Nondiabetic (N) and 3-week streptozotocin (STZ)-induced diabetic (D) male Sprague-Dawley rats (Charles River Laboratories, Saint-Constant, QC, Canada) were used. STZ-injected rats not hyperglycemic within 48 h were excluded. This is an animal model of uncontrolled diabetes and thus is not representative of insulin-treated type 1 diabetic individuals with well-controlled glycemia. Morning (fed) glycemia, body weight, and food intake were measured daily. Experiments were performed in conscious, previously catheterized (left carotid artery and right jugular vein) animals after overnight fasting (16–18 h). On the morning of experimentation, rats were weighed and measured for fasted blood glucose, connected to infusion catheters, and acclimatized for 2 h. Care was taken to avoid stress to the rats because even minimal disturbances can increase the levels of stress hormones that were measured in this study. All procedures were in accordance with Canadian Council on Animal Care Standards and approved by the University of Toronto Animal Care Committee.

**SSTR2a.** This peptide antagonist (PRL-2903, BIM-23458) was synthesized by Dr. David Coy (Tulane University, New Orleans, LA). It has the chemical structure H-Fpa-cyclo[DCys-Pal-DTrp-Lys-Tle-Cys]-Nal-$NH_2$ (22,23) and has a half-maximal inhibitory concentration of 2.5 nmol/L and binds to SSTR2 with a $K_i$ of 26 nmol/L. This peptide was also selective for SSTR2 over SSTR3 and SSTR5 by ~10- and 20-fold, respectively, and had negligible binding affinity to SSTR1 and SSTR4 (23). The antagonist was dissolved in 1% acetic acid and diluted with 0.9% saline.

**Hypoglycemia clamp experiments.** N ($n$ = 14), D ($n$ = 14), and D rats given SSTR2a (D+SSTR2a, $n$ = 18) were used. After basal samples were obtained at $t = -60$ min, infusions of saline or SSTR2a (3,000 nmol/kg/h) were started. Blood glucose was frequently measured (GMD-9D, Analox Instruments USA Inc., Lunenburg, MA). At $t$ = 0, regular insulin (10 units/kg) was injected subcutaneously. Subcutaneous injection rather than intravenous infusion of insulin to induce hypoglycemia mimics the clinical insulin regimen. Also, we have noticed a greater impairment of the glucagon response to hypoglycemia using a subcutaneous insulin bolus injection (13) than with an intravenous infusion (12). The insulin bolus injection method does not result in a steady state of insulin action, making it difficult to measure glucose turnover precisely. Thus, we did not use glucose tracer techniques. A lower insulin dose (5 units/kg) was also studied with the same SSTR2a dose (3,000 nmol/kg/h) in N ($n$ = 5), D ($n$ = 4), and D+SSTR2a ($n$ = 4) groups. The study excluded two rats that did not attain hypoglycemia, and they are not reported in the sample sizes.

Glucose infusions (50% dextrose) were given at a variable rate to clamp glycemia at 2.5 ± 0.5 mmol/L. After $t$ = 180 min, rats were quickly killed by decapitation. A dosing combination of 10 units/kg insulin and 1,500 nmol/kg/h SSTR2a was tested in our pilot work to optimize the antagonist dose. As a control for the effect of the SSTR2a during hypoglycemia in N rats, we measured glucose, glucagon, and corticosterone levels in N rats at the three combination doses of insulin and SSTR2a.

**Control experiments: SSTR2a in the absence of hypoglycemia.** To evaluate the effect of 240 min of SSTR2a infusion per se, glycemia and plasma glucagon, corticosterone, and catecholamines were measured in the absence of hypoglycemia in a separate set of control experiments in N (Ctrl:N, $n$ = 7), D (Ctrl:D, $n$ = 7), and D+SSTR2a rats (Ctrl:D+SSTR2a, $n$ = 7) under basal conditions. In these control experiments, saline rather than insulin was injected at $t$ = 0, and glucose infusions were not used. SSTR2a was infused at 3,000 nmol/kg/h.

**Plasma hormone measurements.** Blood for glucagon (0.3 mL), catecholamines (0.5 mL), insulin and C-peptide (0.3 mL), somatostatin (2 mL), and ACTH (0.5 mL) was collected in chilled tubes containing EDTA (Sangon Ltd. Canada, Scarborough, ON, Canada) and Trasylol (Bayer Canada Ltd., Etobicoke, ON, Canada). Blood for corticosterone (0.1 mL) was collected in heparinized tubes. After plasma was removed, packed erythrocytes were resuspended in heparinized saline (10 USP units/mL) containing 1% BSA and reinfused into the rat. Plasma glucagon and insulin (LINCO Research Inc., St. Charles, MO), catecholamines (LDN GmbH & Co. KG, Nordhorn, Germany), somatostatin (Euro-Diagnostica AB, Malmo, Sweden), corticosterone, and ACTH (MP Biomedicals, Solon, OH) were measured by radioimmunoassay.

**Pancreatic glucagon and somatostatin protein measurements.** Frozen pancreata were homogenized on ice in an extraction medium (9.2% hydrochloric acid, 5% formic acid, 1% trifluoroacetic acid). Protein was isolated by eluting pancreatic homogenate supernatant using C18 Sep-Pak cartridges (Waters Ltd., Milford, MA) with 0.1% trifluoroacetic acid. Hormone measurements were determined using radioimmunoassay and normalized for total protein content by spectrophotometer analysis.

**Data analysis.** One-way ANOVA or repeated measures ANOVA, followed by Duncan's post hoc test, were used as appropriate. Statistics were performed using Statistica software (Statsoft Inc., Tulsa, OK).

## RESULTS

**Daily blood glucose, body weight, and food intake.** Fed morning blood glucose, body weight, and food intake were measured daily. Fed morning glycemia increased fourfold in D compared with N in hypoglycemia ($P < 0.0001$) and control ($P < 0.0001$) studies (Supplementary Table 1). Diabetic groups within each study had similar hyperglycemia leading up to the clamps. Similarly, D had decreased body weight compared with N in the hypoglycemia ($P < 0.0001$) and control ($P < 0.002$) studies and similar body weight as D+SSTR2a (Supplementary Table 1). Average daily food intake also increased in D compared with N in the hypoglycemia ($P < 0.0002$) and control ($P < 0.0001$) studies (Supplementary Table 1).

**Hypoglycemia clamp experiments.** In the high-insulin-dose (10 units/kg) groups, D had elevated basal plasma glucagon (51 ± 5 vs. 32 ± 4 pg/mL; $P < 0.004$), corticosterone (156 ± 26 vs. 42 ± 9 ng/mL; $P < 0.006$), and epinephrine (148 ± 28 vs. 55 ± 11 pg/mL; $P < 0.04$) compared with N, and both groups of D rats had similar basal plasma hormone levels before administration of SSTR2a (D+SSTR2a: basal glucagon, corticosterone, and epinephrine levels were 65 ± 5 pg/mL, 148 ± 33 ng/mL, and 134 ± 57 pg/mL, respectively). Basal plasma insulin was reduced to one-half of the levels in N ($P < 0.05$) in the D and D+SSTR2a groups (Table 1). In the low-insulin-dose (5 units/kg) groups, D also had elevated basal plasma glucagon versus N rats (52 ± 8 vs. 13 ± 6 pg/mL; $P < 0.01$), but the increases in D versus N for basal corticosterone (220 ± 19 vs. 174 ± 38 ng/mL) and epinephrine (93 ± 33 vs. 40 ± 12 pg/mL) and the reduction in basal insulin (0.3 ± 0.2 vs. 0.6 ± 0.4 ng/mL) did not reach statistical significance.

In the hypoglycemia studies, overnight fasting markedly lowered basal blood glucose in diabetic rats in the high-insulin-dose cohort (Fig. 1*A*) and normalized basal blood glucose in diabetic rats in the low-insulin-dose cohort (Fig. 1*B*), but their fed blood glucose levels were markedly elevated (Supplementary Table 1). All groups reached target hypoglycemia (2.5 ± 0.5 mmol/L) by 70 (10 units/kg insulin group; Fig. 1*A*) or 30 min (5 units/kg insulin group; Fig. 1*B*) after insulin injection and remained at hypoglycemia until the end of the experiment at 180 min. In the high-insulin-dose experiments, as expected, N exhibited a robust 11-fold increase in plasma glucagon, whereas D had a markedly attenuated glucagon response ($P < 0.0002$ vs. N; Fig. 2*A*). The glucagon response in D+SSTR2a was fully normalized ($P < 0.0001$ vs. D; Fig. 2*A*). Similarly, the corticosterone response was significantly blunted in D compared with the 10-fold increase observed in N ($P < 0.002$) but was greatly improved in D+SSTR2a ($P < 0.05$ vs. D; Fig. 2*B*). This increase in corticosterone response was mirrored by a tendency of increased plasma ACTH levels in D+SSTR2a (ACTH levels in N: 368 ± 27; D: 210 ± 10; D+SSTR2a: 315 ± 59 pg/mL; D vs. N, $P < 0.01$; D+SSTR2a vs. D, $P$ = 0.07). Glucagon and corticosterone responses of diabetic rats were also enhanced by SSTR2a using lower-dose insulin (5 units/kg; Fig. 3). The peak glucagon response in D+SSTR2a appeared greater than in N, although this was not statistically significant. The parallel trends of plasma ACTH did not reach significance in the low-insulin-dose

TABLE 1
Plasma catecholamine and insulin levels during hypoglycemia clamps with 10 or 5 units/kg insulin and 3,000 nmol/kg/h SSTR2a

| | | Time (min) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | –60 | 0 | 20 | 40 | 60 | 90 | 180 |
| Hypoglycemia clamps using 10 units/kg insulin and 3,000 nmol/kg/h SSTR2a | | | | | | | | |
| EPI (pg/mL) | N | 55 ± 11 | 66 ± 12 | 1,155 ± 427 | 2,908 ± 480 | N/A | 3,790 ± 288 | 1,751 ± 224 |
| | D | 148 ± 28* | 144 ± 30 | 1,058 ± 214 | 1,983 ± 491 | | 3,993 ± 367 | 2,218 ± 312 |
| | D+SSTR2a | 134 ± 57* | 87 ± 35 | 1,201 ± 769 | 2,593 ± 713 | | 3,311 ± 420 | 1,894 ± 478 |
| Hypoglycemia clamps using 5 units/kg insulin and 3,000 nmol/kg/h SSTR2a | | | | | | | | |
| EPI (pg/mL) | N | 40 ± 12 | 133 ± 75 | N/A | N/A | 3,495 ± 554 | N/A | 1,629 ± 510 |
| | D | 93 ± 33 | 75 ± 60 | | | 2,797 ± 1,704 | | 1,606 ± 267 |
| | D+SSTR2a | 147 ± 40 | 141 ± 20 | | | 2,860 ± 644 | | 1,240 ± 132 |
| Hypoglycemia clamps using 10 units/kg insulin and 3,000 nmol/kg/h SSTR2a | | | | | | | | |
| NE (pg/mL) | N | 162 ± 26 | 184 ± 25 | 325 ± 37 | 460 ± 21 | N/A | 654 ± 39 | 811 ± 58 |
| | D | 211 ± 35 | 190 ± 33 | 332 ± 41 | 409 ± 54 | | 702 ± 45 | 787 ± 47 |
| | D+SSTR2a | 334 ± 60 | 305 ± 37 | 429 ± 83 | 524 ± 52 | | 902 ± 104 | 985 ± 43 |
| Hypoglycemia clamps using 5 units/kg insulin and 3,000 nmol/kg/h SSTR2a | | | | | | | | |
| NE (pg/mL) | N | 323 ± 50 | 310 ± 45 | N/A | N/A | 663 ± 43 | N/A | 762 ± 111 |
| | D | 324 ± 29 | 283 ± 44 | | | 1,010 ± 271 | | 794 ± 42 |
| | D+SSTR2a | 282 ± 64 | 221 ± 13 | | | 963 ± 199 | | 957 ± 96 |
| Hypoglycemia clamps using 10 units/kg insulin and 3,000 nmol/kg/h SSTR2a | | | | | | | | |
| INS (ng/mL) | N | 1.0 ± 0.07 | 0.9 ± 0.07 | N/A | N/A | 141.5 ± 8.0 | N/A | 59.6 ± 7.4 |
| | D | 0.5 ± 0.04* | 0.5 ± 0.07* | | | 93.2 ± 7.2* | | 24.1 ± 2.9* |
| | D+SSTR2a | 0.5 ± 0.1* | 0.7 ± 0.2* | | | 107.9 ± 14.1* | | 26.3 ± 4.4* |
| Hypoglycemia clamps using 5 units/kg insulin and 3,000 nmol/kg/h SSTR2a | | | | | | | | |
| INS (ng/mL) | N | 0.6 ± 0.4 | 0.6 ± 0.2 | N/A | N/A | 62.4 ± 13.2 | N/A | 5.2 ± 0.9 |
| | D | 0.3 ± 0.2 | 0.2 ± 0.5 | | | 59.4 ± 17.8 | | 1.5 ± 0.5 |
| | D+SSTR2a | 0.3 ± 0.07 | 0.5 ± 0.2 | | | 58.6 ± 18.0 | | 3.0 ± 1.5 |

Data are presented as means ± SEM. EPI, plasma epinephrine; INS, plasma insulin; NE, plasma norepinephrine. N/A denotes times at which samples for the particular hormone were not obtained. Hypoglycemia experiments using 10 units/kg insulin and 3,000 nmol/kg/h SSTR2a: N, $n = 14$; D, $n = 14$; D+SSTR2a, $n = 18$. Hypoglycemia experiments using 5 units/kg insulin and 3,000 nmol/kg/h SSTR2a: N, $n = 5$; D $n = 4$; D +SSTR2a, $n = 4$. *$P < 0.05$ D, D+SSTR2a vs. N.

study (ACTH levels in N: 486 ± 107; D: 274 ± 71; D+SSTR2a: 465 ± 88 pg/mL).

In our pilot studies, 1,500 nmol/kg/h SSTR2a, a dose based on previous in vivo studies in N rats (22), yielded improvements but not complete restoration of the glucagon and corticosterone responses (Supplementary Table 2). Because a larger dose of SSTR2a could be required to overcome the increased amount somatostatin in the D rats, we doubled the dose of SSTR2a in subsequent experiments. D attained epinephrine levels similar to N, suggesting that in this model, diabetic rats do not exhibit a defect of epinephrine counterregulation (Table 1). SSTR2a did not alter the epinephrine response to hypoglycemia, presumably because the maximal response was already achieved. Norepinephrine responses were likewise similar in all groups (two- to fivefold increases; Table 1). Plasma insulin levels after high-dose (10 units/kg) insulin injection increased to greater absolute levels in N than in D and D+SSTR2a, despite administering the same dose (Table 1). This may be due to differences in body weight. However, insulin levels between D and D+SSTR2a were similar, suggesting that the differences in counterregulatory hormone responses in the diabetic groups result from the SSTR2a and were not due to changes in insulin. In the high-dose insulin hypoglycemia clamps, neither glucose infusion rates nor the total amount of glucose infused to maintain target hypoglycemia (Fig. 2*C*) differed among the groups. However, in the low-dose (5 units/kg) hypoglycemia clamps, D+SSTR2a required significantly lower glucose infusion rates and had a lower total glucose requirement than D ($P < 0.008$, Fig. 3*C*).

**Effect of SSTR2a in N rats during hypoglycemia.** Blood glucose levels during hypoglycemic clamp experiments were the same in N and N+SSTR2a at each of the dosing combinations of insulin to induce hypoglycemia and SSTR2a tested (Fig. 1*D–E* and Supplementary Fig. 1*A*). At our pilot study dose of 1,500 nmol/kg/h, the SSTR2a had no effect on glucagon responses (Supplementary Fig. 1*B*). At 3,000 nmol/kg/h, the SSTR2a decreased plasma glucagon levels in response to hypoglycemia (Figs. 2*D* and 3*D*), an effect in stark contrast to that described in diabetic rats. This suppressive effect of 3,000 nmol/kg/h SSTR2a in N rats was more marked at the higher (10 units/kg, Fig. 2*D*) than at the lower dose of insulin (5 units/kg, Fig. 3*D*). To evaluate whether the antagonist had an effect on insulin secretion, we measured plasma C-peptide levels but could not find any difference (Table 2). SSTR2a had no effect on corticosterone responses to hypoglycemia (Figs. 2*E* and 3*E*).

**Effect of SSTR2a in the absence of hypoglycemia.** All control groups had similar basal hormone levels (Table 3). The lack of difference in glucagon and corticosterone between Ctrl:N and Ctrl:D may be attributed to elevated levels of these basal hormones in Ctrl:N, suggesting some stress. Glycemia remained steady throughout the 4-h experiment (Fig. 1*C*). Notably, glycemia in D+SSTR2a was unaltered. However, Ctrl:D+SSTR2a demonstrated very modest, transient increases in plasma glucagon at 40, 60, and 90 min (Table 3), although area under the curve calculations revealed no significant difference in overall glucagon in Ctrl:D and Ctrl:D+SSTR2a ($P = 0.12$). Plasma corticosterone, epinephrine, and norepinephrine levels also remained similar in all groups, which suggests minimal stress

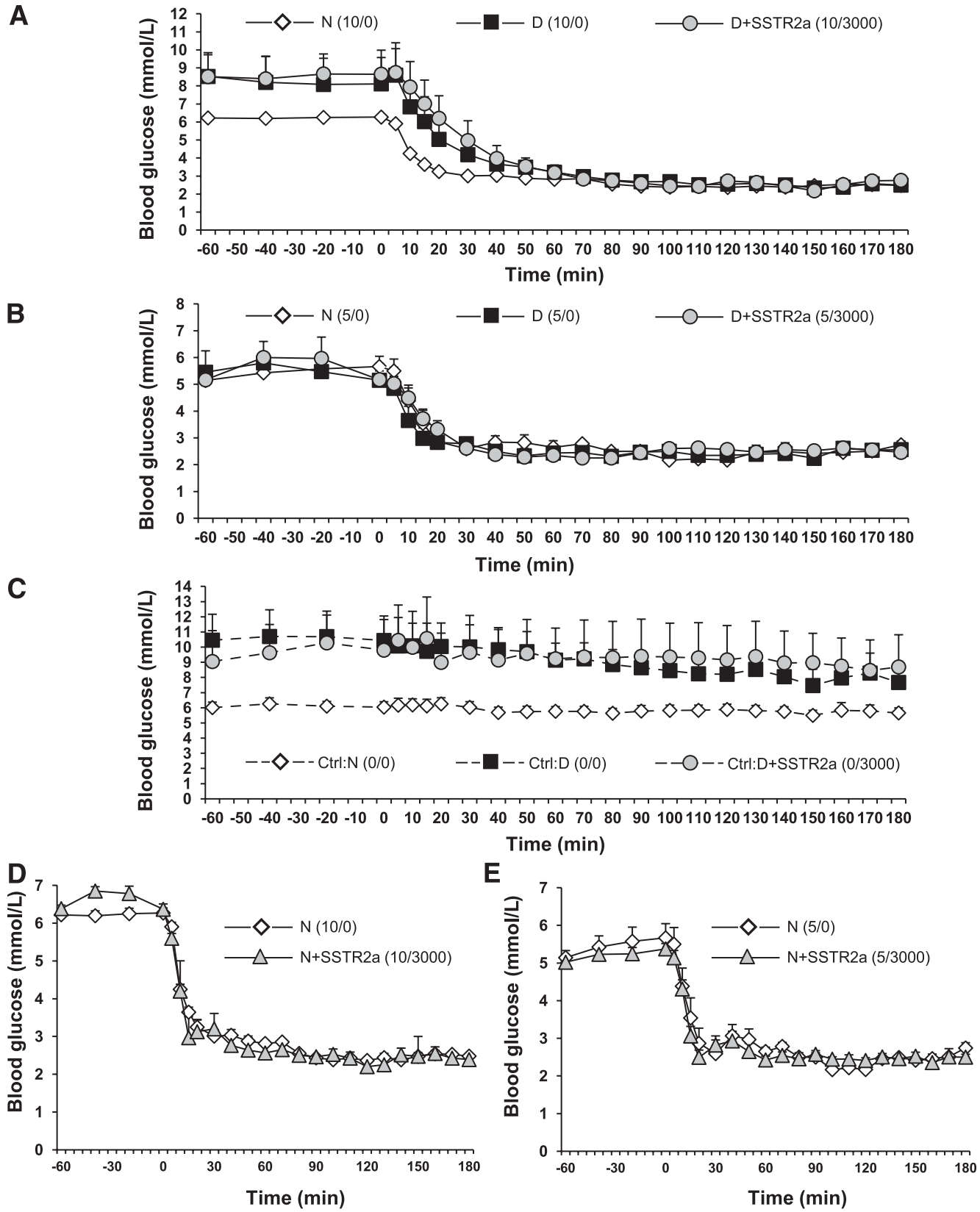


**FIG. 1. Blood glucose levels during (*A*) hypoglycemia clamp (10 units/kg insulin, 3,000 nmol/kg/h SSTR2a), (*B*) hypoglycemia clamp (5 units/kg insulin, 3,000 nmol/kg/h SSTR2a), and in (*C*) control experiments in the absence of insulin (0 units/kg insulin, 3,000 nmol/kg/h SSTR2a). *D*: Hypoglycemia clamp in N rats (10 units/kg insulin, 3,000 nmol/kg/h SSTR2a). *E*: Hypoglycemia clamp in N rats (5 units/kg insulin, 3,000 nmol/kg/h SSTR2a). Bracketed numbers beside group names indicate doses of insulin (units/kg) and SSTR2a (nmol/kg/h). Hypoglycemia experiments using 10 units/kg insulin and 3,000 nmol/kg/h SSTR2a: N, *n* = 14; D, *n* = 14; D+SSTR2a, *n* = 18. Hypoglycemia experiments using 5 units/kg insulin and 3,000 nmol/kg/h SSTR2a: N, *n* = 5; D, *n* = 4; D+SSTR2a, *n* = 4. Control experiments using 0 units/kg insulin and 3,000 nmol/kg/h SSTR2a: N, *n* = 7; D, *n* = 7; D+SSTR2a, *n* = 7. Data are represented as means ± SEM.**

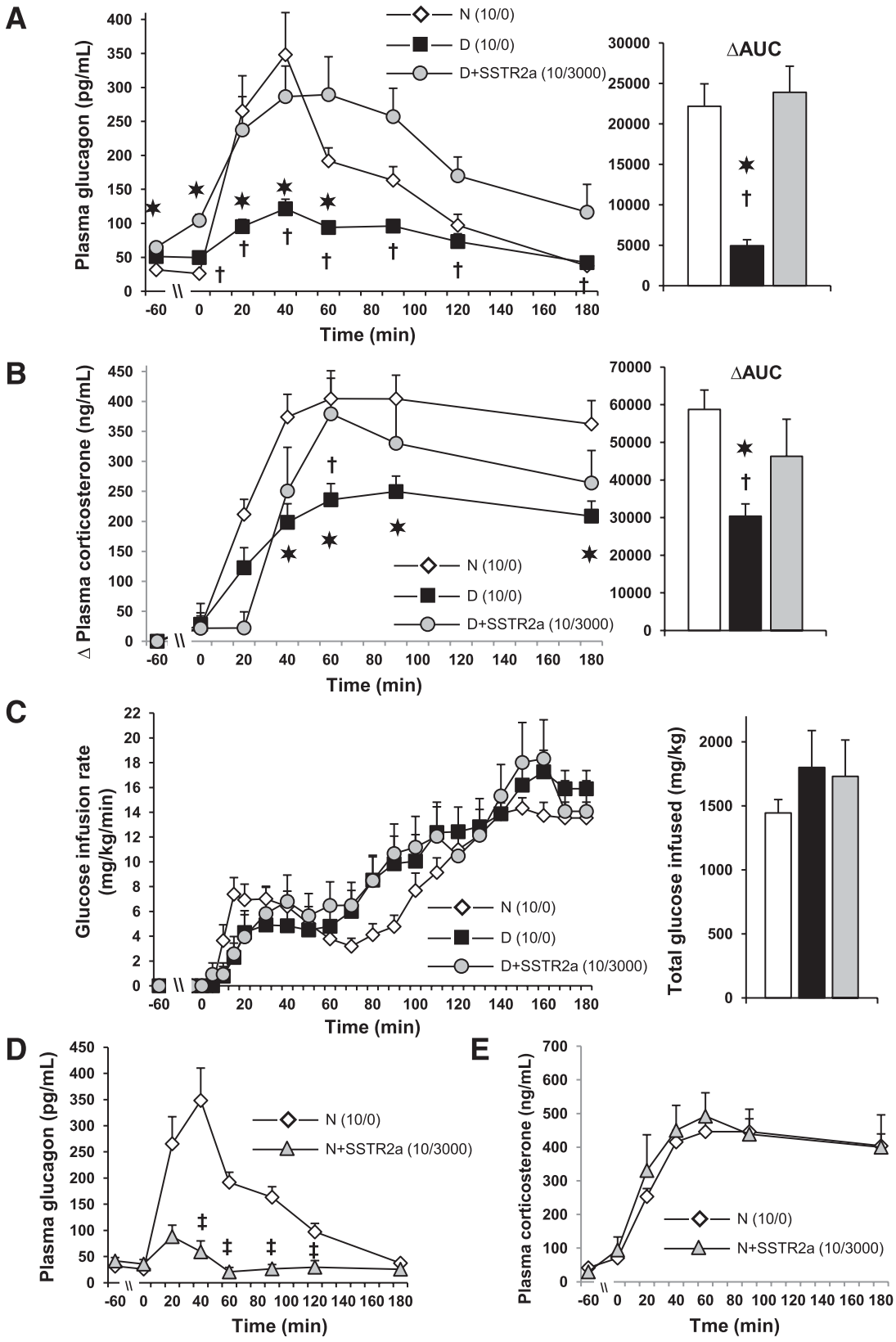


**FIG. 2. Plasma hormone levels and glucose infusion during hypoglycemia clamp (10 units/kg insulin, 3,000 nmol/kg/h SSTR2 antagonist). *A*: Glucagon levels. *B*: Corticosterone responses. *C*: Glucose infusion rate and total glucose infused. *D*: Effect of SSTR2a on plasma glucagon levels in N rats. *E*: Effect of SSTR2a on plasma corticosterone levels in N rats. N, $n$ = 14; D, $n$ = 14; D+SSTR2a, $n$ = 18; N+SSTR2a, $n$ = 4. Bracketed numbers beside group names indicate doses of insulin (units/kg) and SSTR2a (nmol/kg/h). Areas under the curve from baseline (ΔAUC) were calculated using Prism software (GraphPad Software, San Diego, CA). White bars, N; black bars, D; gray bars, D+SSTR2a. Data are represented as means ± SEM. ★$P$ < 0.002 D vs. N; †$P$ < 0.05 D vs. D+SSTR2a; ‡$P$ < 0.05 N vs. N+SSTR2a.**

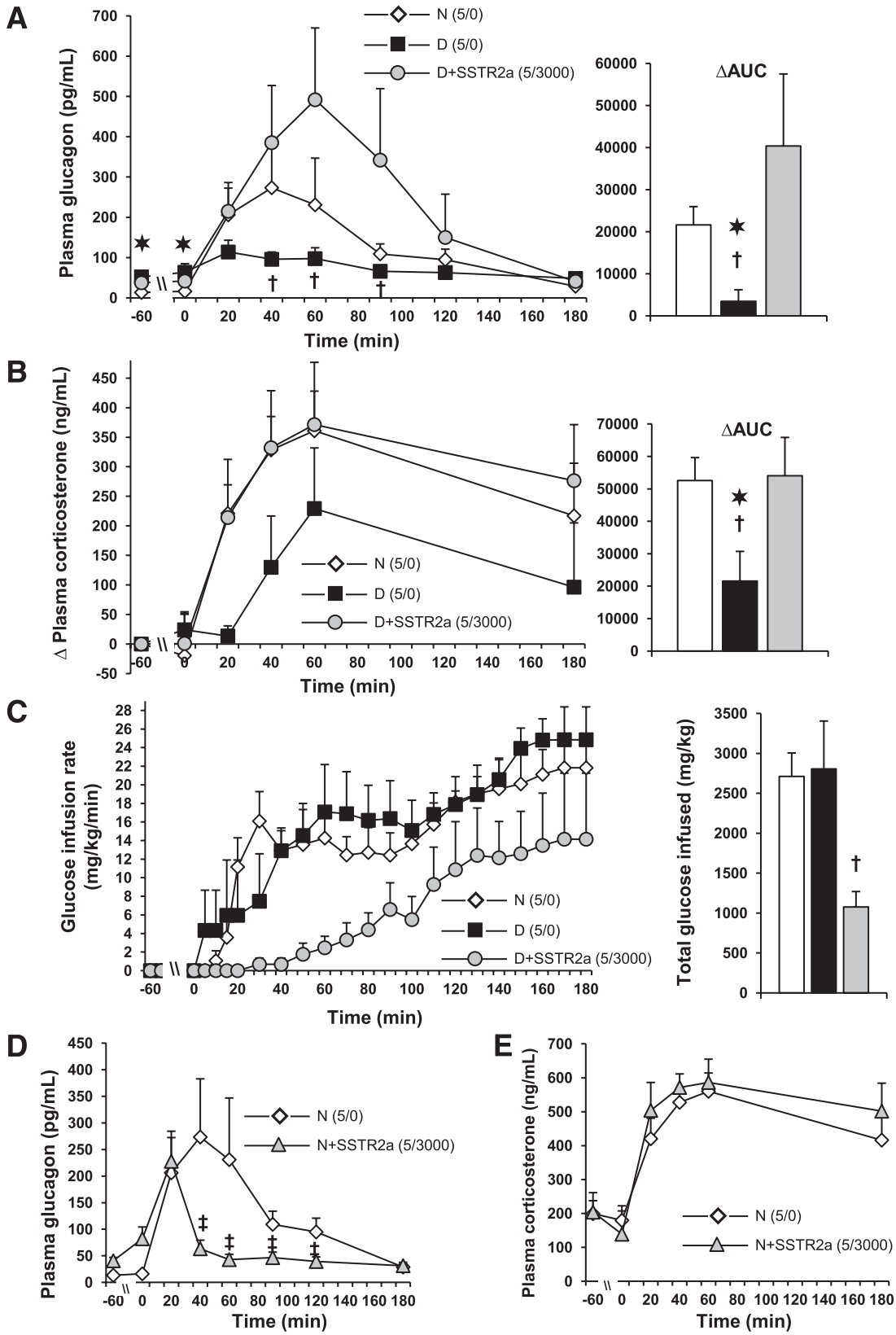


**FIG. 3. Plasma hormone levels and glucose infusion during hypoglycemia clamp (5 units/kg insulin, 3,000 nmol/kg/h SSTR2a). *A*: Glucagon levels. *B*: Corticosterone responses. *C*: Glucose infusion rate and total glucose infused. *D*: Effect of SSTR2a on plasma glucagon levels in N rats. *E*: Effect of SSTR2a on plasma corticosterone levels in N rats. N, $n = 5$; D, $n = 4$; D+SSTR2a, $n = 4$; N+SSTR2a, $n = 10$. Bracketed numbers beside group names indicate doses of insulin (U/kg) and SSTR2a (nmol/kg/h). Areas under the curve from baseline (ΔAUC) were calculated using Prism software (GraphPad Software, San Diego, CA). White bars, N; black bars, D; gray bars, D+SSTR2a. Data are represented as means ± SEM. ★$P < 0.002$ D vs. N; †$P < 0.05$ D vs. D+SSTR2a; ‡$P < 0.05$ N vs. N+SSTR2a.**

TABLE 2
Plasma C-peptide levels in N rats that underwent hypoglycemia clamps using 10 or 5 units/kg insulin and 3,000 nmol/kg/h SSTR2a

| | Time (min) | | | |
|---|---|---|---|---|
| | −60 | 0 | 60 | 180 |
| Plasma C-peptide (nmol/L) | | | | |
| Hypoglycemia clamp using 10 units/kg insulin and 3,000 nmol/kg/h SSTR2a | | | | |
| N ($n$ = 10) | 0.70 ± 0.07 | 0.67 ± 0.08 | 0.06 ± 0.01 | 0.04 ± 0.01 |
| N+SSTR2a ($n$ = 4) | 0.87 ± 0.05 | 0.67 ± 0.08 | 0.09 ± 0.01 | 0.06 ± 0.00 |
| Hypoglycemia clamp using 5 units/kg insulin and 3,000 nmol/kg/h SSTR2a | | | | |
| N ($n$ = 5) | 0.49 ± 0.11 | 0.52 ± 0.10 | 0.13 ± 0.02 | 0.06 ± 0.01 |
| N+SSTR2a ($n$ = 9) | 0.49 ± 0.06 | 0.48 ± 0.09 | 0.08 ± 0.01 | 0.05 ± 0.01 |

Data are presented as means ± SEM.

as a result of the subcutaneous injection of saline (Table 3). Thus, the SSTR2a, per se, did not elicit hyperglycemia or marked sustained elevations of stress hormones.

**Pancreatic glucagon and pancreatic and plasma somatostatin measurements.** Pancreatic glucagon protein content was 50% greater in D compared with N rats in the hypoglycemia and control studies ($P < 0.02$) and was similar in D and D+SSTR2a rats (Fig. 4*A*). Similarly, pancreatic somatostatin protein content after hypoglycemia was greater by 65% in D compared with N rats ($P < 0.02$) and was not affected by SSTR2a (Fig. 4*B*). All three control groups showed statistically similar pancreatic somatostatin content (Ctrl:N vs. Ctrl:D: $P = 0.09$; Ctrl:D vs. Ctrl:D+SSTR2a: $P = 0.07$). Plasma somatostatin concentrations taken at euthanasia showed a similar trend to pancreatic somatostatin: after hypoglycemia, plasma somatostatin increased 62% in D, but this failed to reach statistical significance ($P = 0.08$, Fig. 4*C*). Baseline plasma somatostatin levels before hypoglycemia were not obtained because somatostatin assays require a large amount of plasma. Therefore, samples at euthanasia of control groups that did not experience hypoglycemia served as a baseline comparison.

## DISCUSSION

**Role for SSTR2 in glucagon enhancement.** The glucagon response to hypoglycemia in diabetes is attenuated or abolished (2,3). We demonstrate for the first time that SSTR2 antagonism enhances hypoglycemia-stimulated glucagon and corticosterone release in diabetic rats. The inhibitory effect of somatostatin on α-cell glucagon release via SSTR2 is well established in rodents (16) and humans (17), but this inhibitory effect of somatostatin was not examined during insulin-induced hypoglycemia. Global somatostatin knockout increased nutrient-stimulated, but not basal, glucagon secretion compared with wild-type mice in vivo and in isolated islets, suggesting a role of locally released somatostatin on stimulated, but not basal, glucagon secretion (24). Similarly, isolated islets from SSTR2 knockout mice showed approximately twofold greater stimulated glucagon secretion than wild-type mice (16). In human isolated islets, a dose-dependent reversal of SSTR2a-induced glucagon suppression was achieved by using the same SSTR2a as the current study (25). Thus, our findings using this SSTR2a may also be relevant to humans. We demonstrate that the pancreatic content of glucagon is elevated

TABLE 3
Plasma hormone levels during control experiments in the absence of insulin and 3,000 nmol/kg/h SSTR2a

| | Time (min) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | −60 | 0 | 20 | 40 | 60 | 90 | 120 | 180 |
| Plasma glucagon (pg/mL) | | | | | | | | |
| Ctrl:N | 50 ± 6 | 70 ± 15 | 79 ± 24 | 64 ± 14 | 42 ± 12 | 47 ± 9 | 33 ± 9 | 39 ± 8 |
| Ctrl:D | 45 ± 3 | 50 ± 12 | 41 ± 13 | 43 ± 8 | 50 ± 10 | 51 ± 18 | 55 ± 10 | 59 ± 17 |
| Ctrl:D+SSTR2a | 50 ± 4 | 88 ± 18 | 101 ± 24 | 100 ± 22** | 97 ± 15** | 95 ± 11** | 80 ± 13 | 73 ± 13 |
| Plasma corticosterone (ng/mL) | | | | | | | | |
| Ctrl:N | 165 ± 31 | 210 ± 47 | 313 ± 36 | 294 ± 60 | 233 ± 56 | 195 ± 38 | N/A | 208 ± 29 |
| Ctrl:D | 205 ± 49 | 248 ± 75 | 351 ± 93 | 349 ± 84 | 335 ± 86 | 254 ± 74 | | 180 ± 43 |
| Ctrl:D+SSTR2a | 273 ± 46 | 261 ± 53 | 316 ± 49 | 322 ± 55 | 331 ± 58 | 293 ± 50 | | 225 ± 40 |
| Plasma epinephrine (pg/mL) | | | | | | | | |
| Ctrl:N | 31 ± 15 | 94 ± 19 | 89 ± 16 | 112 ± 19 | 85 ± 14 | 82 ± 25 | N/A | 45 ± 12 |
| Ctrl:D | 110 ± 46 | 85 ± 31 | 157 ± 43 | 116 ± 29 | 264 ± 112 | 382 ± 272 | | 123 ± 49 |
| Ctrl:D+SSTR2a | 217 ± 65 | 180 ± 85 | 243 ± 156 | 385 ± 191 | 345 ± 149 | 371 ± 163 | | 235 ± 111 |
| Plasma norepinephrine (pg/mL) | | | | | | | | |
| Ctrl:N | 356 ± 39 | 393 ± 45 | 428 ± 46 | 435 ± 88 | 353 ± 79 | 392 ± 78 | N/A | 293 ± 41 |
| Ctrl:D | 310 ± 82 | 292 ± 59 | 272 ± 31 | 310 ± 48 | 376 ± 96 | 301 ± 80 | | 246 ± 108 |
| Ctrl:D+SSTR2a | 290 ± 38 | 238 ± 58 | 242 ± 100 | 381 ± 125 | 351 ± 76 | 315 ± 96 | | 330 ± 105 |
| Plasma insulin (ng/mL) | | | | | | | | |
| Ctrl:N | 1.1 ± 0.2 | 1.1 ± 0.1 | N/A | N/A | 0.8 ± 0.1 | N/A | N/A | 0.8 ± 0.1 |
| Ctrl:D | 0.7 ± 0.1* | 0.7 ± 0.1 | | | 0.7 ± 0.1 | | | 0.7 ± 0.1 |
| Ctrl:D+SSTR2a | 0.5 ± 0.1 | 0.7 ± 0.2 | | | 0.6 ± 0.2 | | | 0.6 ± 0.1 |

Data are presented as means ± SEM. N/A denotes times at which samples for the particular hormone were not obtained. Control experiments using 0 units/kg insulin and 3,000 nmol/kg/h SSTR2a: N, $n$ = 7; D, $n$ = 7; D+SSTR2a, $n$ = 7. *$P < 0.05$ D vs. N; **$P < 0.03$ D vs. D+SSTR2a.

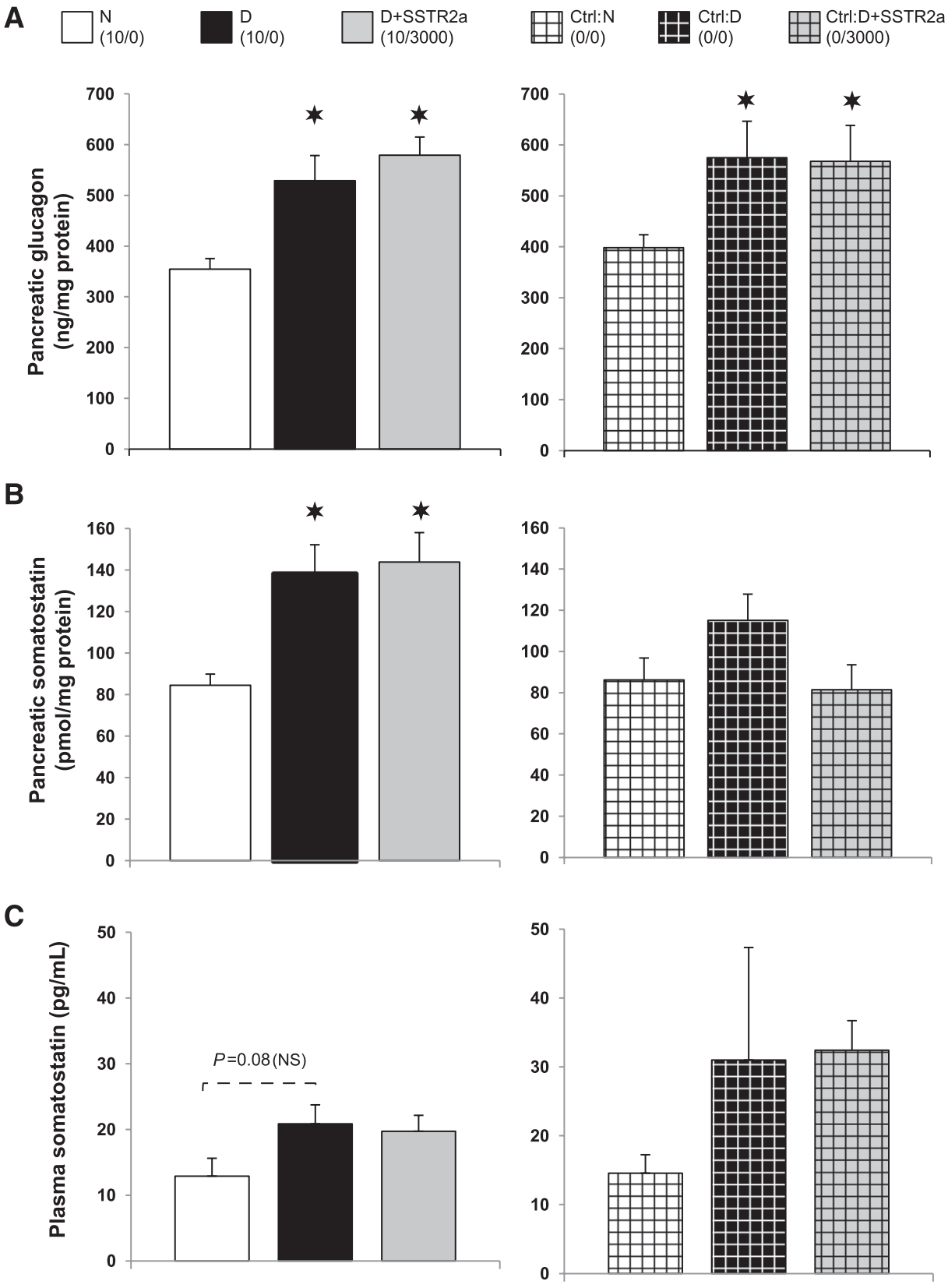


**FIG. 4. Pancreatic protein content of glucagon (*A*) and somatostatin (*B*) and plasma somatostatin (*C*) after hypoglycemia clamp (10 units/kg insulin, 3,000 nmol/kg/h SSTR2a) and control experiments in the absence of insulin (0 units/kg, 3,000 nmol/kg/h). Solid bars in the *left panels* represent hypoglycemia experiments. Hashed bars in the *right panels* represent control experiments. Bracketed numbers beside group names indicate doses of insulin (U/kg) and SSTR2a (nmol/kg/h). Hypoglycemia experiments using 10 units/kg insulin and 3,000 nmol/kg/h SSTR2a: N, $n$ = 14; D, $n$ = 14; D+SSTR2a, $n$ = 18. Control experiments using 0 units/kg insulin and 3,000 nmol/kg/h SSTR2a: N, $n$ = 7; D, $n$ = 7; D+SSTR2a, $n$ = 7. Data are represented as means ± SEM. ★$P < 0.05$ D, D+SSTR2a vs. N.**

in diabetic rats even subsequent to hypoglycemia. This suggests that during hypoglycemia, the defect in glucagon secretion is not due to a reduction of pancreatic glucagon content, but there is a defect in the secretory mechanism of the α-cell. We hypothesize that the secretory defect of the α-cell is at least partly due to increased pancreatic somatostatin. We speculate that inhibition of the α-cell by somatostatin during hypoglycemia may decrease glucagon release in diabetic but not in nondiabetic rats because of increased pancreatic somatostatin, because of altered islet architecture in diabetes (see below), and possibly because of increased sensitivity of the α-cell to large amounts of insulin. Islet somatostatin release can be mediated by a glucose-dependent mechanism (26), and whether this also applies to diabetic islets remains to be explored. Paradoxically, a twofold larger dose of SSTR2a in N rats even attenuated rather than enhanced glucagon release. We could not demonstrate enhanced insulin release from plasma C-peptide levels. Thus, it is unlikely that this paradoxic effect could have been mediated by a stimulated release of insulin resulting from a nonspecific effect of a high dose of the SSTR2a on other somatostatin receptors on β-cells (which express SSTR5 and not SSTR2 in rats), which could locally suppress glucagon release. Although a local paracrine effect cannot be excluded, it is possible that our peptide antagonist could have partial agonist activity at high doses when the normal α-cell is not exposed to sufficient amounts of endogenous competing somatostatin molecules. However, future in vitro studies may be necessary to unravel the mechanism underlying this paradox.

It was questioned whether somatostatin plays a role during hypoglycemia because somatostatin-secreting δ-cells are downstream of glucagon-secreting α-cells in the islet microcirculation of nondiabetic rats (27). However, δ-cells in diabetic rats are also distributed in central portions of islet cells because the architecture of islet cell type is altered (28), suggesting that paracrine actions of islet hormones are altered in diabetes such that somatostatin release upstream of α-cells may affect glucagon secretion. The arrangement of human endocrine islet cells is likewise more disperse throughout the islet, which provides evidence for the proximity of δ-cells and α-cells (29–31). Furthermore, paracrine signaling may also occur via diffusion within the islet interstitium, independently of blood flow.

One cannot exclude that mild stress due to diabetic metabolic derangements, which increased levels of some basal hormones, may affect hormone counterregulation to hypoglycemia. However, both diabetic groups had similar plasma hormone levels at baseline, and thus the effects due to SSTR2a treatment can be compared.

**Role for SSTR2 in corticosterone enhancement.** The glucocorticoid (corticosterone in rodents or cortisol in humans) response to hypoglycemia can be impaired in diabetes (12,32–34). Dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis can contribute to defective counterregulation because it regulates not only glucocorticoid but also, potentially, epinephrine secretion (32,35). Presently, we demonstrate that peripheral administration of the SSTR2a normalizes the attenuated corticosterone response to hypoglycemia in D rats in the high- (10 units/kg) and low- (5 units/kg) insulin-dose studies, irrespective of basal corticosterone, which was elevated (high-insulin-dose cohort) or similar (low-insulin-dose cohort) to N rats.

The role of somatostatin on the regulation of corticosterone is less well documented than its role on suppressing glucagon release. Corticosterone levels are elevated in somatostatin knockout mice (35), which supports the role of endogenous somatostatin regulating corticosterone release. Somatostatin inhibits the secretion of corticotropin-releasing hormone and ACTH (16). SSTR2 has been localized in several brain regions, and specifically in areas of HPA activity—the hypothalamic paraventricular nucleus (31)—and ACTH cells in the anterior pituitary (32). Plasma ACTH, measured at the end of 180 min of hypoglycemia, was decreased in D compared with N controls in the high-insulin-dose study. At sacrifice there was a tendency for ACTH to be increased in the D+SSTR2a rats. This could indicate that this antagonist may have affected the HPA axis centrally or at the level of the pituitary, but this should have been evaluated at the peak of ACTH release. However, it is also possible that the SSTR2a affects corticosterone release directly at the level of the adrenal gland where SSTR2 is expressed (36,37).

**SSTR2a does not cause hyperglycemia or undesired stress.** Because somatostatin antagonists may worsen glycemia by augmenting glucagon release in diabetic patients, it was important to evaluate the effect of the SSTR2a during the basal state. Presently, we demonstrate that the SSTR2a administered for 4 h during basal conditions in vivo yielded only very small transient increases of glucagon, which did not cause hyperglycemia and did not alter corticosterone or catecholamine levels. The fact that SSTR2 antagonism does not elicit large increases in glucagon release during basal conditions may be explained as follows: Somatostatin receptor–coupled signaling in the α-cell via SSTR2 inhibits $Ca^{2+}$-induced glucagon exocytosis via $G_{i2}$-dependent activation of calcineurin, which "deprimes" glucagon-containing granules associated with *L*-type $Ca^{2+}$ channels, which are associated with stimulated glucagon secretion. It has less effect on the granules associated with *N*-type $Ca^{2+}$ channels, which mediate basal glucagon secretion (38,39).

In addition to inhibiting exocytosis, somatostatin can also suppress glucagon release by inhibiting α-cell electrical activity (40). Furthermore, hormones such as glucagon-like peptide 1 can regulate glucagon release independent of glucose levels, insulin, or somatostatin, at least in vitro (41). In islets of nondiabetic mice, SSTR2a enhanced glucagon secretion during low-glucose stimulation but had no effect at 7 mmol/L glucose (42).

However, it remains to be investigated whether prolonged administration of the SSTR2a will lead to hyperglycemia. SSTR2 knockout mice showed no differences in fasting and fed glycemia, fasting and fed plasma glucagon levels, or responses to glucose tolerance tests compared with wild-type mice. However, diet-induced obesity in these knockout mice caused fasting and fed hyperglycemia, fed hyperglucagonemia, and impaired glucose tolerance compared with wild-type mice given the same high-fat diet (43). It has not been explored whether SSTR2 antagonism in diabetic subjects will enhance glucagon release in response to other stimuli known to trigger glucagon release such as amino acid–rich meals or exercise.

**Glucose requirement during hypoglycemia.** Despite using half the dose of insulin in our low-dose versus high-dose insulin experiments, glucose infusion rates are greater in groups induced with 5 units/kg insulin. This could reflect a greater insulin sensitivity of these rats, as indicated by their lower basal glycemia, and perhaps to the slightly lower overall counterregulatory hormone response, which may reflect a smaller glycemic decrease or less brain insulin.

The low-insulin-dose rats may have been selected for their insulin sensitivity because rats that did not reach hypoglycemia were excluded.

At the high insulin dose, there were no differences in glucose infusions rates between N and D rats and between D+SSTR2a and D rats. However, glucose infusion was decreased with SSTR2 antagonism during low-dose insulin–induced hypoglycemia. A lower glucose requirement during hypoglycemia is consistent with normalized glucagon and corticosterone responses in the D+SSTR2a group. We cannot explain the difference between the high- and low-insulin-dose groups. Perhaps this effect is due to the glucagon response in the SSTR2a-treated group, which appears to be greater in the low-insulin-dose cohort, by a greater effect of glucagon under conditions of higher insulin sensitivity (as in insulin-treated type 1 diabetes), and/or by the effect of high-dose insulin, which overcomes the effect of glucagon on the liver to impact the glucose requirement. Future studies to evaluate the efficacy of SSTR2 antagonism on enhancing glucose production during hypoglycemia in diabetic subjects under various insulin conditions could address this issue.

In conclusion, we demonstrate that glucagon and corticosterone hormone responses to hypoglycemia can be normalized in diabetic rats with specific antagonism of SSTR2, which does not elicit hyperglycemia or substantial elevations in these hormones during basal conditions. Our results therefore suggest an important role for pancreatic and, especially, increased pancreatic somatostatin, and possibly circulating or brain somatostatin, in defective glucagon and corticosterone counterregulation in diabetes. This does not rule out other mechanisms of impairment. In N rats, SSTR2a did not augment (or paradoxically even decreased) the glucagon response, indicating that in contrast to D rats, somatostatin is not an important inhibitor of glucagon release during hypoglycemia. If the threat of hypoglycemia can be decreased or prevented, intensive insulin treatment would be more effective.

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