Somatostatin Receptor Type 2 (SSTR2) Antagonism and Hypoglycemia in Diabetes

by

Jessica Tin Yan Yue

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Graduate Department of Physiology
University of Toronto

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Abstract

Hypoglycemia is one of the most serious acute complications in intensively treated diabetes. Recurrent hypoglycemia predisposes individuals to subsequent hypoglycemia, and diminished counterregulatory hormone responses increase this threat. Elevated pancreatic and/or circulating somatostatin has been reported in diabetic humans and animals, and we postulated that excessive somatostatin contributes to the attenuation of counterregulatory hormone release during hypoglycemia in diabetes. It is known that somatostatin suppresses stimulated secretion of glucagon, epinephrine, and corticosterone. We hypothesized that selective somatostatin receptor type 2 (SSTR2) antagonism would: (Study 1) improve hormone counterregulation to hypoglycemia, and (Study 2) ameliorate hypoglycemia in recurrently hypoglycemic rats. Using both high (10 U/kg) and low (5 U/kg) dose insulin to induce hypoglycemia, we demonstrate that inhibiting the action of somatostatin on SSTR2 normalizes the severely attenuated glucagon and corticosterone responses to acute hypoglycemia in diabetic rats. These improvements were specific to
diabetes since SSTR2 antagonist did not increase these hormones in non-diabetic rats in response to hypoglycemia. In the absence of hypoglycemia, SSTR2 antagonist neither markedly alters glycemia nor causes sustained elevations in counterregulatory hormones in diabetic animals. Diabetic rats exhibit up to 65% and 75% more pancreatic and plasma somatostatin than non-diabetic rats following hypoglycemia, respectively. Despite improvements of glucagon and corticosterone, expression of gluconeogenic enzymes PEPCK1 and G6Pase was unaltered. SSTR2 antagonism reduced the glucose requirement during a hypoglycemic clamp induced with a lower dose of insulin. In recurrently hypoglycemic diabetic rats, we demonstrate that SSTR2 antagonist treatment reduces the depth and duration of hypoglycemia and promotes the recovery to euglycemia, without affecting the glycemia-lowering effect of insulin. This amelioration of hypoglycemia by SSTR2 antagonism may be attributable in part to the observed modest improvements of glucagon, epinephrine, and corticosterone counterregulation following recurrent hypoglycemia. These results implicate an important role for increased pancreatic, and possibly circulating, somatostatin in defective hypoglycemic counterregulation in diabetes.
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Lastly and most importantly, I would like to acknowledge my family for their unceasing support and encouragement in all that I do, whether I deserve it not. To my sister and brother-in-law, Josephine and Karwin – thank you for always helping to keep my spirits up and for keeping me real. And especially to my parents, Suzanna and Edwin – I thank you for your constant affirmation, your prayers, and your love. Thank you for continually teaching me and reminding me to always keep my focus on the most important things in life. Truly, the impact of the lessons I have learned at home far surpass the knowledge acquired from my studies.

Jessica T.Y. Yue
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<table>
<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ACCORD</td>
<td>Action to Control Cardiovascular Risk in Diabetes</td>
</tr>
<tr>
<td>ACTH</td>
<td>adrenocorticotropic hormone</td>
</tr>
<tr>
<td>ADVANCE</td>
<td>Action in Diabetes and Vascular Disease</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>AP</td>
<td>area postrema</td>
</tr>
<tr>
<td>ARC</td>
<td>arcuate nucleus</td>
</tr>
<tr>
<td>AUC</td>
<td>area under the curve</td>
</tr>
<tr>
<td>AVP</td>
<td>arginine vasopressin</td>
</tr>
<tr>
<td>BLA</td>
<td>basolateral amygdala</td>
</tr>
<tr>
<td>BNST</td>
<td>bed nucleus of the stria terminalis</td>
</tr>
<tr>
<td>CPE</td>
<td>carboxypeptidase E</td>
</tr>
<tr>
<td>CRH</td>
<td>corticotrophin-releasing hormone</td>
</tr>
<tr>
<td>DCCT</td>
<td>Diabetes Control and Complications Trial</td>
</tr>
<tr>
<td>DMH</td>
<td>dorsomedial nucleus</td>
</tr>
<tr>
<td>DMNV</td>
<td>dorsal motor nucleus of the vagus</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediamine tetraacetic acid</td>
</tr>
<tr>
<td>GABA</td>
<td>gamma-aminobutyric acid</td>
</tr>
<tr>
<td>GE</td>
<td>glucose-excited</td>
</tr>
<tr>
<td>GHRH</td>
<td>growth hormone releasing hormone</td>
</tr>
<tr>
<td>GI</td>
<td>glucose-inhibited</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
</tr>
<tr>
<td>HAAF</td>
<td>hypoglycemia-associated autonomic failure</td>
</tr>
<tr>
<td>HPA</td>
<td>hypothalamic-pituitary-adrenal</td>
</tr>
<tr>
<td>IML</td>
<td>intermediolateral column</td>
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<tr>
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<tr>
<td>iv</td>
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<tr>
<td>LC</td>
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<tr>
<td>LHA</td>
<td>lateral hypothalamic area</td>
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<tr>
<td>MPO</td>
<td>medial preoptic area</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>NOD</td>
<td>non-obese diabetic</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
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<tr>
<td><strong>NTS</strong></td>
<td>nucleus of the solitary tract</td>
</tr>
<tr>
<td><strong>PBN</strong></td>
<td>parabrachial nucleus</td>
</tr>
<tr>
<td><strong>PC1/2</strong></td>
<td>prohormone convertase-1/-2</td>
</tr>
<tr>
<td><strong>PCR</strong></td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td><strong>PMV</strong></td>
<td>portal-mesenteric veins</td>
</tr>
<tr>
<td><strong>POMC</strong></td>
<td>proopiomelanocortin</td>
</tr>
<tr>
<td><strong>PVN</strong></td>
<td>paraventricular nucleus</td>
</tr>
<tr>
<td><strong>RIA</strong></td>
<td>radioimmunoassay</td>
</tr>
<tr>
<td><strong>RVLM</strong></td>
<td>rostroventrolateral medulla</td>
</tr>
<tr>
<td><strong>sc</strong></td>
<td>subcutaneous</td>
</tr>
<tr>
<td><strong>SCN</strong></td>
<td>suprachiasmatic nucleus</td>
</tr>
<tr>
<td><strong>SEM</strong></td>
<td>standard error of the mean</td>
</tr>
<tr>
<td><strong>SON</strong></td>
<td>supraoptic nucleus</td>
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<tr>
<td><strong>SSTR1/2/3/4/5</strong></td>
<td>somatostatin receptor type 1/2/3/4/5</td>
</tr>
<tr>
<td><strong>STZ</strong></td>
<td>streptozotocin</td>
</tr>
<tr>
<td><strong>UKPDS</strong></td>
<td>United Kingdom Prospective Diabetes Study</td>
</tr>
<tr>
<td><strong>VADT</strong></td>
<td>Veterans Affairs Diabetes Trial</td>
</tr>
<tr>
<td><strong>VMH</strong></td>
<td>ventromedial hypothalamus</td>
</tr>
<tr>
<td><strong>VMN</strong></td>
<td>ventromedial hypothalamic nucleus</td>
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1 Introduction

1.1 Hypoglycemia

Hypoglycemia is a perturbation to physiological homeostasis and has potentially devastating consequences. Hypoglycemia is defined as an “abnormally low plasma glucose concentration that expose[s] the individual to potential harm” by the American Diabetes Association Workgroup on Hypoglycemia, and quantitatively, it is a measured plasma glucose level of ≤3.9 mM (1). Glucose is the predominant fuel to the brain (2). When arterial plasma glucose levels fall below the physiological euglycemic range (i.e. 3.9 – 6.1 mM), glucose transport to the brain becomes rate limiting to brain glucose metabolism (3;4). Thus, it is imperative that blood glucose concentrations are maintained above this level for brain function and ultimately for survival (3;4).

Symptoms arising from iatrogenic hypoglycemia are conventionally classified into two main categories: i) neuroglycopenic, and ii) neurogenic or autonomic (5). Neuroglycopenic symptoms stem from glucose deprivation of the brain and include behavioural changes, fatigue, and cognitive impairments, and confusion (5). Restoration of blood glucose levels results in recovery from these deficits in brain function (6). In severe neuroglycopenia, seizures, loss of consciousness, and brain death can ensue, but this is rare and associated with profound and prolonged hypoglycemia (7). Neurogenic symptoms arise from the autonomic nervous system reacting to the perception of hypoglycemia. Adrenergic...
neurogenic symptoms mediated by catecholamines include shakiness/tremor, a pounding heart, and nervousness/anxiety, while cholinergic neurogenic symptoms mediated by acetylcholine include sweatiness, hunger, and a tingling sensation (8). In healthy individuals, these symptoms of hypoglycemia often do not present until plasma glucose levels reach a threshold of approximately 3.0 mM (9-11). Neurogenic symptoms are present (3.2 mM) before neuroglycopenic symptoms (2.8 mM) and cognitive impairment (2.7 mM) (12).

1.1.1 Normal sensing of hypoglycemia: sites of glucose detection

Hypoglycemia is a fairly uncommon event except in individuals that use glucose-lowering medications, particularly insulin and sulphonylureas or other insulin secretagogues to treat diabetes, or in patients with insulinomas (11). This is because the body has several physiological defense mechanisms to counter decreases in blood glucose to restore blood glucose from hypoglycemia to euglycemia. In healthy subjects, hypoglycemia elicits a characteristic succession of responses. Glucose sensors are responsible for the detection of glycemic decline and initiate an orchestrated series of responses mediated via neural networks that integrate the various afferent inputs and instigate efferent responses, particularly as they relate to hypoglycemia. Glucose sensors important for glucose counterregulation are found in three primary areas: the hepatic portal-mesenteric veins (PMV), the hypothalamus, and the brainstem (13;14).

The portal vein can also sense a decrease in plasma glucose via a GLUT2-dependent glucose sensing mechanism and relays this signal to the central nervous system via a vagus nerve afferent (15-18). It has been demonstrated that normalizing glucose levels in the PMV can suppress hypoglycemia-induced food intake (19) and sympathoadrenal responses (20;21).
Hepatoportal glucose sensors also stimulate glucose storage in the liver, muscle, and adipose tissue (22).

In the hypothalamus, established key areas involved in glucose detection include the ventromedial hypothalamus (VMH) (specifically the ventromedial hypothalamic nuclei (VMN) and arcuate nuclei (ARC)) (23-26), lateral hypothalamic area (LHA) (27-29), and paraventricular nuclei (PVN) (30-32) in which glucose sensing neurons have been located. The VMH integrates forebrain neuronal input with ascending information from the brainstem and then sends output to regions involved in control of various behaviours and physiologic responses (33). These hypothalamic areas identified for glucose sensing also have critical roles for glucagon and catecholamine counterregulation in response to hypoglycemia (34-40), which will be discussed below.

In the brainstem, glucose sensing neurons are abundant in the nucleus of the solitary tract (NTS) (15;41-43), area postrema (AP) (44), and dorsal motor nucleus of the vagus (DMNV) (45). These glucose sensing regions of the brainstem have been implicated in the regulation of glucose homeostasis (46-48).

More recently, nuclei in the medial amygdala have also been implicated as an important glucose sensing area that also elicits counterregulatory responses (49). Detailed mechanisms involved with sensing hypoglycemia and neuronal integration have been recently reviewed (13;14;18). In summary, perturbation of glucose homeostasis triggers central integration of the various glucose-detection signals, and an autonomic response is initiated that activates sympathetic (neural and adrenomedullary) and parasympathetic outflow (50). Taken together, these counterregulatory responses involve inhibition of
endogenous insulin secretion and activation of several hormonal and neuroendocrine systems to restore blood glucose to euglycemia.

1.1.2 Normal counterregulatory hormone responses to hypoglycemia

1.1.2.1 Insulin.

First and foremost, with declining blood glucose levels, insulin secretion must cease (51;52), and this alleviates both its suppressive effect on hepatic glucose production and its stimulatory effect of glucose utilization in muscle and adipose tissue. Reduced insulin secretion also lowers insulin-mediated inhibition of lipolysis and protein catabolism and stimulation of lipogenesis and protein anabolism, which results in increased plasma levels of free fatty acids, glycerol, and amino acids, the products of which can serve as substrates or energy sources for gluconeogenesis (53;54). Reduced insulin secretion is believed to be due to a direct effect of hypoglycemia on pancreatic β-cells (51;55). Typically, the decrease in pancreatic insulin secretion occurs when the blood glucose threshold reaches 4.6 mM (3). Sympathoadrenal activation in the form of norepinephrine from pancreatic sympathetic nerves and epinephrine from the adrenal medulla may also play a role in inhibiting insulin secretion via α2-adrenergic stimulation (56). It should be noted that the catecholamines can also stimulate insulin via β2-adrenergic receptors, but the α2-adrenergic effect appears to predominant, at least in human islets (57).

1.1.2.2 Glucagon.

In addition to the decrement in insulin, several other counterregulatory hormones work together to normalize blood glucose levels in response to hypoglycemia. It is well known that glucagon is key in restoring euglycemia during acute hypoglycemia (58;59). Glucose recovery from hypoglycemia is impaired by approximately 40% when glucagon secretion is inhibited by somatostatin infusion (60). This dependence on the glucagon response for
hypoglycemic recovery is true for both short-term (59-61) and prolonged (62) episodes of hypoglycemia. Glucagon helps to restore euglycemia by stimulating hepatic glucose production via glycogenolysis, or it favours gluconeogenesis when precursors (i.e. lactate, amino acids, and glycerol) are abundant (63).

Increments in glucagon secretion can result from direct effects of hypoglycemia on pancreatic \( \alpha \)-cells (64). The glycemic threshold of approximately 3.8 mM triggers glucagon release (9;10). Other mechanisms of the secretory glucagon response to hypoglycemia include increased autonomic inputs (i.e. sympathetic neural, parasympathetic neural, and adrenomedullary) and decreased intraislet insulin (50). During hypoglycemia, the sympathetic (65;66) and parasympathetic (67;68) nerves and the adrenal medulla are activated – releasing norepinephrine (69;70), acetylcholine (71), and epinephrine (72;73), respectively – all of which result in increased glucagon release. Catecholamines stimulate \( \alpha \)-cell glucagon secretion via \( \alpha_2 \)- and \( \beta_2 \)-adrenergic receptors (69;74;75), whereas acetylcholine acts via M\(_1\) and M\(_3\) muscarinic receptors (76;77). Decreased intraislet insulin secretion during hypoglycemia is believed to be one of the signals involved in glucagon secretion (78-80). More recent work has suggested that a decrement in zinc, a co-secretory product of insulin release, and not necessarily insulin itself, provides the trigger to initiate glucagon secretion during hypoglycemia since zinc acts on \( K_{atp} \)-channels to hyperpolarize \( \alpha \)-cells (81;82), but this view has been challenged (83). \( \gamma \)-aminobutyric acid (GABA), which is co-localized in \( \beta \)-cells with insulin, also suppresses glucagon release (84). Functional GABA\(_\alpha\) receptors are expressed on \( \alpha \)-cells, and activation of these receptors by GABA secreted by \( \beta \)-cells mediates chloride ion influx thereby hyperpolarizing the \( \alpha \)-cell and inhibiting glucagon release (85-87). Intraislet somatostatin exerts a tonic suppressive effect on glucagon release (88;89). It was first postulated nearly 20 years ago that relative
abundance of pancreatic somatostatin in diabetic dogs may contribute to the poor glucagon response to hypoglycemia (90), and this concept will be discussed in detail throughout this thesis. Ghrelin is another peptide predominantly produced by the stomach but which is also synthesized in small amounts in the pancreatic islet. The ghrelin receptor is widely expressed in the adult rat islet and has been co-localized with glucagon-positive cells (91). Locally released ghrelin could have a paracrine effect on \( \alpha \)-cell glucagon release, perhaps promoting glucagon release during hypoglycemia. However, studies in perfused rat pancreas indicate that concentrations of ghrelin within the physiological range (0.5–3 nM) had no effect on basal or arginine-stimulated glucagon release, despite mildly inhibiting both stimulated insulin and somatostatin release (92). It has also been postulated that the \( \alpha \)-cell may sense hypoglycemia directly and secrete glucagon in response to low glucose concentrations as has been observed in the perfused isolated pancreas (93). However, lowering glucose levels in isolated \( \alpha \)-cells failed to stimulate glucagon secretion (94), which suggested no direct role of hypoglycemia on stimulating glucagon release. More recent evidence suggests that the direct stimulatory effect of low glucose on glucagon secretion by the \( \alpha \)-cell may be of less significance as compared to the stimulatory influences of other factors (95;96). This is supported by the intraislet insulin hypothesis which involves a “switch-off” concept in which the \( \beta \)-cell reduces insulin secretion as a key mechanism to initiate the glucagon response to hypoglycemia (80;97). This concept as it pertains to diabetes will be discussed in section 1.4.1.

In addition to these factors, central nervous system regulation of glucagon release has also been demonstrated. Lesioning of the VMH caused 50-60% and 75-80% decreases in glucagon and catecholamine responses during mild (3.0 mM) and severe (2.5 mM) hypoglycemia, respectively (34). Glucagon secretion can be induced with local
glucoprivation in the VMH by direct 2-deoxyglucose injection (35), and hypoglycemia-induced glucagon secretion can be suppressed by direct VMH injection of glucose (36). Furthermore, glutamate release by neurons of the VMH has also been implicated as an important mechanism in glucagon counterregulation to hypoglycemia (98). The VMH has been shown to be part of the neurocircuitry that links sympathetic and parasympathetic innervation of the pancreas to regulate glucagon release, and Buijs et al. reports other key brain centres including the suprachiasmatic nucleus (SCN), ARC, dorsomedial nucleus (DMH), locus coeruleus (LC), NTS, and DMNV and intermediolateral column (IML) of the spinal cord to be involved in this neurocircuitry (99).

1.1.2.3 Epinephrine.

Epinephrine has an important role in augmenting blood glucose levels, although the epinephrine response may not be critical in correcting hypoglycemia unless glucagon secretion is deficient (59;100;101), which often occurs in type 1 diabetes or long-standing type 2 diabetes. This will be further discussed below. It has been suggested that in type 1 diabetes, a robust epinephrine response can compensate for an absent glucagon response (102). Hypoglycemia is one of the most potent stimulators of epinephrine release from the adrenal medulla (103) and has been demonstrated to elevate plasma epinephrine levels by more than 60-fold (103;104). Hypoglycemia activates sympathetic nervous activity in muscle (105;106) and the pancreas (65;67). The release of epinephrine is triggered when arterialized venous blood glucose levels fall to approximately 3.8 mM, which is similar to the glycemic threshold for glucagon secretion (9;10). Epinephrine both stimulates glucose production as well as limits glucose utilization directly by inhibiting glucose uptake by peripheral tissues and indirectly by suppressing insulin release (107). Epinephrine stimulates hepatic glycogenolysis via $\alpha_1$- and $\beta_2$-adrenergic receptors (108).
stimulates hepatic, and to some extent renal, gluconeogenesis, also by mobilizing
gluconeogenic substrates. The effect of epinephrine to reduce glucose uptake involves
limiting glucose utilization by insulin-sensitive tissues via direct effects and also by
increasing non-esterified fatty acids levels. Catecholamine release from the adrenal gland
can also stimulate synthesis of glucocorticoids (109).

1.1.2.4 Norepinephrine.
Although some norepinephrine is also released by the adrenal medulla (approximately 20%
of its total catecholamine release is norepinephrine), the primary source of circulating
norepinephrine is from sympathetic nerves innervating blood vessels. Normally, most of the
norepinephrine released by sympathetic nerves is taken back up by the nerves (some is also
taken up by extra-neuronal tissues) where it is metabolized. Thus, only a small amount of
norepinephrine enters the blood into the peripheral circulation. However, at times of high
sympathetic nerve activation, the amount of norepinephrine entering the blood increases
dramatically (108). Norepinephrine has also been shown to increase blood glucose levels
during hypoglycemia via mechanisms similar to those of epinephrine (110). Yet during
hypoglycemia, plasma norepinephrine levels are lower compared with epinephrine (i.e.
approximately 5-fold as compared to 60-fold elevations of epinephrine) (103;111). During
hypoglycemia, norepinephrine is released not only from sympathetic nerve terminals, but
also in smaller quantities from the adrenal medulla (112-114). Increases in norepinephrine
within muscle and adipose tissue are observed in response to hypoglycemia implicating
activation of sympathetic nerves in these glucoregulatory tissues. In muscle,
norepinephrine and epinephrine can stimulate glycogenolysis and release lactate via a β2-
adrenal mechanism and decrease insulin-stimulated glycogen synthesis (108). In adipose
tissue, norepinephrine and epinephrine mobilize free fatty acids by stimulating lipolysis also mediated through β-adrenergic receptors (115;116).

1.1.2.5 *Growth hormone and glucocorticoids.* Suppression of insulin secretion and activation of glucagon and epinephrine secretions are the primary defenses to safeguard against hypoglycemia. Growth hormone and glucocorticoids also help to promote the restoration of euglycemia during prolonged hypoglycemia (i.e. ≥ 3 hours) (64;117;118), whereas their effects during short term hypoglycemia are not as strong. Growth hormone and glucocorticoid responses are triggered at glycemic thresholds of approximately 3.7 and 3.2 mM, respectively (9;10). Growth hormone and glucocorticoids – cortisol in humans and corticosterone in rodents – act by decreasing glucose utilization and supporting glucose production (117;118). During prolonged hypoglycemia, the lack of either growth hormone or glucocorticoid counterregulation results in a hypoglycemia of greater severity which even enhanced glucagon or epinephrine counterregulation cannot overcome (117;118). Growth hormone secretion from the anterior pituitary is primarily regulated by growth hormone release hormone (GHRH) and somatostatin release from the hypothalamus. Insulin-induced hypoglycemia, L-dopa administration, and arginine infusion indirectly stimulate growth hormone secretion, and one study has suggested that unlike the other stimuli, hypoglycemia can elicit increases in growth hormone secretion independently of GHRH and somatostatin regulation (119). During hypoglycemia, an increase in growth hormone is involved in enhancing glucose production, limiting glucose utilization, and stimulating lipolysis to increase circulating plasma free fatty acids, glycerol, and β-hydroxybutyrate, which can provide gluconeogenic substrates and well as fuels to spare glucose (117).
In response to hypoglycemia, the hypothalamic-pituitary-adrenal (HPA) axis is activated, and this results in the release of cortisol/corticosterone from the adrenal cortex (120). Activation of the HPA axis involves the release of corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP) from neurons of the paraventricular nucleus of the hypothalamus into the median eminence. Here, these neuropeptides travel via the hypophyseal portal circulation to the anterior pituitary where they stimulate the production of adrenocorticotropic hormone (ACTH) from its precursor, proopiomelanocortin (POMC). ACTH is released into the peripheral circulation where it acts on the adrenal cortex to release cortisol/corticosterone, which ultimately inhibit further HPA activity (121;122). This negative feedback control is mediated by cortisol/corticosterone acting on glucocorticoid receptors (GRs) in the hippocampus, PVN, and anterior pituitary (for suppressing the stress response) or on mineralocorticoid receptors at the hippocampus (for tonic basal inhibition of the HPA axis) (123). During hypoglycemia, glucocorticoids act on glucocorticoid receptors on the liver to stimulate glucose production directly by enhancing gluconeogenic enzymes and indirectly by increasing the pool of readily available substrates (124). Glucocorticoids reduce insulin-mediated glucose uptake and utilization by muscle and adipose tissue (125;126). In support of the primary counterregulatory defenses, glucocorticoids can potentiate the effects of glucagon and epinephrine on the liver and suppress insulin’s inhibitory effect of glucose production (127;128).

1.1.2.6 Glucose autoregulation.

Glucose autoregulation is endogenous glucose production independent of the other aforementioned hormones and neural glucoregulatory mechanisms, and it is inversely proportional to ambient plasma glucose levels (50). This has been observed in humans during marked hypoglycemia (110;129). It is only at very severe hypoglycemia (i.e. 1.7 –
1.9 mM) that glucose autoregulation may occur (129;130). It is believed that glucose autoregulation plays a relatively minor role in glucose counterregulation (3).

1.2 Hypoglycemia in diabetes

1.2.1 Clinical Significance – Hypoglycemia in type 1 diabetes mellitus

Hypoglycemia is rare in people without diabetes (11;63;131-133). However, hypoglycemia is one of the most serious acute complications of well-controlled type 1 diabetic patients and often represents the limiting factor to insulin therapy (134-140). Based on conservative estimates, 10% of type 1 diabetic individuals using conventional insulin therapy and 25% of diabetic individuals using intensive insulin therapy experience at least one bout of severe, debilitating hypoglycemia, often with seizure or coma, in a given year (138;139;141). Furthermore, patients practicing conventional insulin therapy undergo an average of one bout of mild to moderate symptomatic hypoglycemia per week while those being intensively treated suffer from two such episodes weekly (142). It can thus be projected that over 40 years of type 1 diabetes, an average patient may experience 2000-4000 bouts of symptomatic hypoglycemia (143). Hence, iatrogenic hypoglycemia is evidently problematic for type 1 diabetic patients attempting to manage their plasma glucose levels in a range commonly recommended by practitioners to curtail diabetic complications resulting from poor glycemic control. To further complicate matters, the strongest predictor of severe hypoglycemia was a history of severe hypoglycemia (144). This suggests any bout of hypoglycemia can predispose an individual to suffer subsequent bouts of hypoglycemia. The topic of recurrent hypoglycemia will be discussed below.
1.2.2 Clinical Significance – Hypoglycemia in type 2 diabetes mellitus

Individuals with type 2 diabetes generally experience less frequent severe hypoglycemia than those with type 1 diabetes as has been reported by two separate clinical trials (143). Individuals with type 2 diabetes are at a two- to four-fold greater risk of serious cardiovascular outcomes compared with those without diabetes (145). As with type 1 diabetes, the ultimate goal of well-controlled glycemia to reduce the risk of diabetic complications is still elusive due to an increased threat of hypoglycemia that accompanies intensive treatment (146). Treatment-induced hypoglycemia is a limiting factor of glycemic management not only in individuals with type 1 diabetes, but also in many with advanced type 2 diabetic individuals in which there is an absolute endogenous insulin deficiency (63;147-149). Overall, hypoglycemia is less frequent in type 2 diabetes than in type 1 diabetes, but hypoglycemia becomes progressively more frequent and limiting to glycemic control in advanced type 2 diabetes (4). Patients with type 2 diabetes recently started on insulin had rates of hypoglycemia which were similar to those patients who were taking sulphonylureas (150). However, individuals with type 2 diabetes treated with insulin for over 5 years and whose endogenous insulin production as measured by stimulated C-peptide was low had significantly higher rates of hypoglycaemia than individuals with shorter duration of insulin treatment or individuals treated with sulphonylureas, including severe episodes (150;151). Strict glycemic control remains the most effective strategy to reduce the development and/or progression of microvascular disease. Interestingly, however, recent clinical trials in type 2 diabetic patients – Action to Control Cardiovascular Risk in Diabetes Study Group (ACCORD) (152), Action in Diabetes and Vascular Disease (ADVANCE) (153), and Veterans Affairs Diabetes Trial (VADT) (154) – reported that reducing HbA1C to below 7% did not significantly reduce cardiovascular mortality. However, it has been suggested that the study duration of these aforementioned trials may
have been too short to show a significant effect of intensive glycemic control on macrovascular complications (146). The UKPDS study, on the other hand, showed significantly lower relative risk reduction for myocardial infarctions and overall mortality in patients that receive intensive treatment compared with those who received conventional treatment in the 10-year follow-up study (155). Thus, the effect of intensive control of glycemia to reduce cardiovascular morbidity has been demonstrated, but it may be limited to younger patients with shorter duration of diabetes and no prior cardiovascular disease (156).

Despite the benefits of well-controlled glycemia on cardiovascular morbidity, intensive treatment to control glucose is associated with a significantly greater incidence of severe hypoglycemic events (152-154). Mild hypoglycemia events were also more frequent in the intensive control arm as compared to conventional control arm in the ADVANCE trial (153). In the ACCORD trial, there was a 3-fold greater incidence of severe hypoglycemia in the intensive treatment group as compared with conventional treatment group (152). The VADT study reported 3-fold greater incidence of hypoglycemia with symptoms, and a 5-fold greater incidence of hypoglycemia without symptoms, in the intensive versus standard therapy arms (154). This is problematic since in the VADT, severe hypoglycemia was the most powerful predictor of cardiovascular death. Likewise in ACCORD, patients with severe hypoglycemia had higher mortality than those without in both the intensive and conventional treatment study arms. Hypoglycemia is associated with activation of adrenergic responses and prolonged QTc intervals and cardiac arrhythmias (157), which can contribute to increased cardiovascular mortality. In addition, diabetic autonomic neuropathy may also render patients more vulnerable to adverse cardiovascular outcomes (158). Thus, intensive treatment to keep HbA1C below 7% reduces the risk of
macrovascular complications in individuals with type 2 diabetes, but the corollary is that intensive glucose control is associated with frequent episodes of severe hypoglycemia and consequent cardiovascular events leading to mortality.

1.3 Defective counterregulation in diabetes
Normal counterregulatory responses to hypoglycemia have been described above as it applies to healthy individuals. However, hypoglycemia in type 1 diabetic patients is the consequence of an interplay between insulin excess as well as compromised glucose counterregulation (159). In type 2 diabetes, both a history of previous hypoglycemia and duration of insulin treatment are significant predictors of hypoglycemia (148;160). As patients progress with type 2 diabetes, they approach the insulin-deficient end of this disease until they reach the stage of being absolutely endogenous insulin deficient. Then patients are thus similar to individuals with type 1 diabetes in that compromised glucose counterregulation may include defective hormone responses to restore euglycemia, hypoglycemia unawareness, and elevated glycemic thresholds (i.e. lower glycemia required) for responses to hypoglycemia (161).

1.4 Mechanisms of defective counterregulation in diabetes
Fundamental alterations in glucose counterregulation occur in individuals with established type 1 and type 2 diabetes (159;162). When diabetes is fully developed, endogenous insulin secretion in completely absent (163;164), and plasma insulin concentrations reflect that those which are provided by exogenously administered insulin and do not diminish when blood glucose levels fall. Thus the first defense against hypoglycemia, the decrement
of endogenous insulin secretion, is non-existent (159;162). As patients with type 2 diabetes approach the insulin-deficient end of this disease, they become similar to individuals with type 1 diabetes in that both have absolute β-cell failure, which causes the loss of the insulin decrement in response to hypoglycemia (4).

1.4.1 **Glucagon counterregulation in impaired in diabetes**

The glucagon response, the second defense to combat hypoglycemia, is also lost in established type 1 diabetes (165;166) and in advanced type 2 diabetes (167;168). In type 1 diabetes, this usually occurs within the first few years of onset of the disease (165;166). This defect may be specific to insulin-induced hypoglycemia as a stressor since glucagon counterregulation to other stressors, such as arginine infusion or intracerebroventricular carbachol infusion, in diabetic subjects or animals are still intact (166;169;170). Although it has been shown that the impairment is linked to a loss of residual β-cell function (171), the exact mechanisms underlying the defect in glucagon response still remain unclear.

Hypotheses related to impaired glucagon counterregulation as a result of loss of β-cell function include a requirement of a decrement of intraislet insulin release (172;173) as well as a β-cell insulin “switch-off” hypothesis (82;97) to trigger glucagon secretion in response to hypoglycemia. Other hypotheses include chronic hyperglycemia since it was demonstrated that restoration of normal glycemia independent of insulin can improve glucagon counterregulation (174), increased α-cell sensitivity to exogenous insulin as a result of the absence of β-cell function (175), and a defect in autonomic response to hypoglycemia (175), and in particular defective sympathetic innervation of the islet to release glucagon in response to hypoglycemia (176). Reduced autonomic responses to hypoglycemia may be the result of up-regulated brain glucose transport since it has been demonstrated that this is a prominent mechanism after long repeated exposure to
hypoglycemia (177;178). In β-cell-deficient STZ-diabetic rats treated with insulin, it was demonstrated that the glucagon response to insulin-induced hypoglycemia can be enhanced by pancreatic infusion and subsequent switch-off of somatostatin (at hypoglycemia), thereby supporting the notion that glucagon counterregulation is a general disinhibition phenomenon and can be enhanced by switch-off of α-cell-suppressing intrapancreatic signals other than insulin or its co-secretory products (179).

In type 2 diabetes, which tends to be a more heterogeneous disease than type 1 diabetes, investigations of how hypoglycemic counterregulation is altered in this disease must also consider factors such as age, duration of diabetes, β-cell reserve, type of therapy (i.e. diet, exercise, oral medications, insulin, etc.), body fat composition, co-morbidities, and metabolic control (180;181). The glucagon response to hypoglycemia in type 2 diabetes has been shown to be preserved in some studies (182-185), but defective in other reports (167;168;186-188). An absolute insulin deficiency associated with advanced type 2 diabetes is suggested to underlie the glucagon impairment in advanced type 2 diabetes (4;161). A study by Segel et al. separated the glucagon responses of type 2 diabetic subjects on insulin therapy, who represent an insulin-deficient group, from type 2 diabetic subjects on oral medications, who represent the insulin-sufficient group, and demonstrates that insulin-deficient individuals, but not those with endogenous insulin, have diminished glucagon counterregulation to hypoglycemia (168).

Besides these hypotheses of local factors responsible for impaired glucagon counterregulation, several mechanisms related to the central nervous system have also been posited. Local glucopenia in the absence of peripheral hypoglycemia induced by 2-deoxyglucose injection into the VMH stimulates glucagon and epinephrine responses in non-diabetic rats, but these responses were markedly suppressed in diabetic rats (189). It is
speculated that the impairment of the glucagon response to hypoglycemia in diabetes in which there is an absolute insulin deficiency might result in part from the simultaneous increase in insulin at the levels of the VMH and in the pancreatic islet as a result of exogenous insulin administration (190). Recently, it was demonstrated that insulin microinjected into the VMH can suppress the glucagon response to hypoglycemia induced by phloridzin (i.e. hypoglycemia not induced by insulin) by approximately 40% and that VMH blockade of insulin via insulin immunoneutralization can amplify the glucagon response by more than 2-fold (190). However, this has yet to be demonstrated in a diabetic model. The role of glucose in the VMH has also been implicated as mechanism of influencing hypoglycemic counterregulation. Local delivery of glucose into the VMH suppresses glucagon and epinephrine responses to hypoglycemia (36;191). When glucose levels increase in the VMH, an increased in the ATP:ADP ratio in local glucose-excited neurons leads to closure of $K_\text{ATP}$ channels, membrane depolarization, $Ca^{2+}$ influx via voltage-dependent calcium channels, and release of neurotransmitters such as GABA (39;192;193). GABA acting in the VMH blunts glucagon and epinephrine counterregulation to hypoglycemia, whereas inhibiting GABA actions can stimulate these counterregulatory responses (39). A recent study provided the link between the effects of VMH glucose and GABA by demonstrating the glucose administrated in the VMH dose-dependently activates local GABA release, which consequently dose-dependently suppresses glucagon and epinephrine responses to hypoglycemia (191). Studies have also demonstrated a role for a different population of glucose-sensing neurons of the VMH, the glucose-inhibited (GI) neurons, in glucagon counterregulation. GI neurons increase their activity in response to low glucose levels (194). AMP-activated protein kinase (AMPK) acts as an intracellular fuel gauge which responds to a rise in the intracellular AMP:ATP ratio and is thus implicated in the glucose sensing pathway used by GI neurons (195). Activation of AMPK in the VMH with
locally administered 5-aminoimidazole-4-carboxamide (AICAR) lowered the exogenous glucose infusion rate and increased glucose production in response to hypoglycemia in non-diabetic rats (196) and amplified the glucagon responses to hypoglycemia in diabetic rats (197).

1.4.2 Epinephrine counterregulation in diabetes

The epinephrine response to hypoglycemia is critical in a setting of deficient glucagon counterregulation to hypoglycemia (4). Individuals lacking both glucagon and epinephrine counterregulation are at a 25-fold or greater risk of severe hypoglycemia compared to individuals lacking glucagon but have an intact epinephrine response (198;199). A combined defect in both glucagon and epinephrine responses is associated with impaired glucose production in the liver and kidney (143;200). Epinephrine counterregulation to hypoglycemia has been shown to be both impaired (165;201-205) or not impaired (206-208) in type 1 diabetes. In non-diabetic humans in which two different doses of insulin were used to induce hypoglycemia of equivalent depth, it was shown that the higher insulin dose resulted in a markedly greater increase in epinephrine release than the lower dose (209). However, this stimulatory effect of high insulin dose did not amplify epinephrine counterregulation in type 1 diabetic humans, suggesting some adrenomedullary deficit with insulin-deficient diabetes (210). It has been shown that the glycemic threshold for epinephrine release is altered such that lower glycemia is needed to elicit a response (203;211). In type 2 diabetic subjects, the epinephrine response has mostly been reported to be normal (185;188;212). Interestingly, some have also reported an increased in epinephrine counterregulation to hypoglycemia in type 2 diabetic subjects (186;213). This enhanced epinephrine response to hypoglycemia in type 2 diabetes may partially offset impaired glucagon secretion and counteract the effects of hyperinsulinemia on liver, fat, and
skeletal muscle (186) and/or contribute to the promotion of hypoglycemic recovery since an increase in renal glucose release was also observed in this group (213). The factor of age should also be considered since it has been reported in non-diabetic individuals that the glucose threshold for epinephrine and glucagon responses in older patients (2.8 mM) was significantly lower than in younger patients where the threshold was 3.3 mM (214). Indeed, although epinephrine and cortisol responses to hypoglycemia in older type 2 diabetic adults were increased, glucagon and growth hormone responses were reduced when compared to age-matched non-diabetic controls (215). Furthermore, epinephrine defect in diabetes may be stressor-specific: although epinephrine was reduced in response to hypoglycemia and cold stress (216), it was not impaired in response to exercise (204;217). It has also been shown that poorly controlled diabetic individuals may not suffer from impaired epinephrine to the same extent of intensively treated diabetic patients (206;207;211;218), suggesting that prior glycemic control of diabetes may also affect epinephrine counterregulation. In type 2 diabetic patients, for example, intensive treatment to better control glycemia in has been shown to blunt epinephrine counterregulation to hypoglycemia (212;219). However, since tight control of blood glucose levels is also associated with increased frequency of hypoglycemic episodes, it is also possible that recurrent hypoglycemia leads to the impaired epinephrine response observed in diabetes, as has been previously observed (220).

Mechanisms related to the regulation of epinephrine counterregulation by the brain, in particular by the VMH, have been discussed above as it pertains to glucagon. Many of these studies demonstrate simultaneous influences on both glucagon and epinephrine release in both non-diabetic (36;39) and diabetic rats (189), suggesting that the counterregulatory release of both these glucoregulatory hormones are regulated by similar neural pathways integrated by the VMH. Interestingly, VMH administration of insulin did not affect
epinephrine counterregulation (190), suggesting that different neural pathways involving the VMH also exist. Furthermore, it was demonstrated that inhibition of VMH nitric oxide (NO) signalling impairs epinephrine, but not glucagon, counterregulation and results in a greater exogenous glucose infusion rate to prevent hypoglycemia (221). VMH GI neurons respond to decreased glucose by activating AMPK and stimulating the production of NO (195;222), and it has been hypothesized that VMH NO production is necessary for generation of hypoglycemic counterregulation (221).

1.4.3 Norepinephrine, growth hormone, and glucocorticoid counterregulation in diabetes

With respect to the other counterregulatory hormones, it is controversial as to whether or not neuroendocrine responses are impaired in type 1 diabetes. Studies examining norepinephrine counterregulation are varied as some studies find decreased responses (205;223;224) while others do not (202-204;206;225-227). Growth hormone responses to hypoglycemia have also been reported to be decreased (204;224;228;229) or unchanged (203;225-227) in type 1 diabetes. Growth hormone responses to hypoglycemia in type 1 (230) and type 2 (215) diabetic individuals were reduced when compared to age-matched non-diabetic controls. Lastly, glucocorticoid counterregulation has also been reported to be decreased (202;205;206;224;226;228) or unchanged (203;204;225;227). Interestingly, it has been reported that scrupulous avoidance of hypoglycemia could reverse the impairments of some counterregulatory hormone responses (224). Similar to epinephrine, prior strict glycemic control, and consequently repeated hypoglycemia, may also affect the impairment of these responses (206;207;231).
1.4.4 Hypoglycemia unawareness in diabetes

Part of the defect in glucose counterregulation to hypoglycemia in diabetes is the clinical syndrome of hypoglycemia unawareness, which is the impaired awareness of hypoglycemia (4). More specifically, it is the inability to recognize the neurogenic warning symptoms of hypoglycemia or the failure for symptoms to occur before the start of neuroglycopenia (232). With this absence of awareness, a subject developing hypoglycemia is averted from undertaking protective action to prevent or correct impending hypoglycemia, such as nutrient ingestion. Hypoglycemia unawareness is associated with a 6-fold increased risk of severe hypoglycemia (233). It is suggested that the pathophysiology of glucose counterregulation is similar in type 1 and advanced type 2 diabetes although the timeframe of these impairments may be different (4). The failure of β-cell function is more rapid in type 1 diabetes but occurs more slowly in type 2 diabetes, and thus the syndromes of defective glucose counterregulation occur earlier in the former and later in the latter (4).

The attenuation of the sympathoadrenal (mainly sympathetic neural) response causes hypoglycemia unawareness (233), but several factors are believed to contribute to this impairment (234). Hypoglycemia unawareness can result from diminished autonomic responsiveness to hypoglycemia since patients with type 1 diabetes were reported to have reduced β-adrenergic sensitivity, which may play a role to their inability to recognize adrenergic warning signs to hypoglycemia (235). The lack of the catecholamine response to hypoglycemia has been attributed to hypoglycemia unawareness since subjects with this hormone impairment also were asymptomatic for hypoglycemia (236). Elderly patients are more susceptible to hypoglycemia unawareness than middle-aged patients with type 2 diabetes, and this was found to be independent of differences in hormone counterregulation between both groups (237). However, the younger cohort had better autonomic and
neuroglycopenic symptom scores and were better able to estimate their glycemia to be low during hypoglycemia. Prior and repeated hypoglycemia also leads to hypoglycemia unawareness, and it was demonstrated that careful avoidance of hypoglycemia can reverse hypoglycemia unawareness and improve the epinephrine response (238-240). This may be associated with improved β-adrenergic activity as a result of strict avoidance of hypoglycemia as has been observed in type 1 diabetic subjects (241).

Hypoglycemia, even if asymptomatic, causes a vicious cycle of recurrent hypoglycemia by causing hypoglycemia-associated autonomic failure, the clinical syndromes of defective glucose counterregulation and hypoglycemia unawareness (4), as described below.

1.4.5 Hypoglycemia-associated autonomic failure (HAAF) in diabetes

Hypoglycemia-associated autonomic failure, or HAAF, in diabetes is a concept developed by Cryer based on the well-established theories that recent antecedent hypoglycemia (150;153;242), as well as sleep (56) and antecedent exercise (243), cause reduced sympathoadrenal responses to hypoglycemia, which in turn leads to reduced sympathetic neural responses and hypoglycemia unawareness as well as defective glucose counterregulation. Taken together, these impairments lead to a vicious cycle of recurrent hypoglycemia (63). This concept was originally developed to explain the increased susceptibility of hypoglycemia in patients with type 1 diabetes (244) and has since also been applied to explain the increased development of hypoglycemia in advanced type 2 diabetic individuals (168). In type 2 diabetic subjects, recent and repeated hypoglycemia shifts the glycemic thresholds for autonomic (including adrenomedullary epinephrine) and symptomatic responses to subsequent hypoglycemia to lower plasma glucose concentrations, thus contributing to HAAF in type 2 diabetes (168). The concept of HAAF is
widely accepted and quoted by many others with respect to both type 1 and type 2 diabetes (181;219;245-247).

1.5 Recurrent hypoglycemia in non-diabetic subjects

Recurrent hypoglycemia has been shown to blunt subsequent counterregulation to hypoglycemia in healthy, non-diabetic subjects (248-251). For example, it has been consistently demonstrated that glucagon and epinephrine responses to hypoglycemia are diminished after recurrent hypoglycemia in non-diabetic humans (248;250-254) and rats (255). Prior bouts of hypoglycemia blunted epinephrine, but not norepinephrine, responses (111;256). Prior bouts also impaired cognitive function during subsequent hypoglycemia in non-diabetic rats (254;257). Whether impairments exist in the other neuroendocrine counterregulation, however, is debatable. Norepinephrine (248;251) and cortisol (248;250) responses have been reported to be reduced in some studies, but these findings have been inconsistent.

1.6 Recurrent hypoglycemia in diabetes

Recurrent hypoglycemia increases the susceptibility of individuals to subsequent bouts of hypoglycemia since it contributes to both defective hormone counterregulation and reduced symptom recognition (hypoglycemia unawareness) (4). HAAF, as explained above, is relevant in both type 1 diabetes (203) and type 2 diabetes of long duration in which there is an absolute endogenous insulin deficiency (168). In addition to these physiological consequences, hypoglycemia can also have a profound impact on the lives of individuals with diabetes, and it has been noted that this population may fear hypoglycemia more than
the long-term diabetic complications (1;258). As such, the management of glycemia in diabetes is impeded by the threat of iatrogenic hypoglycemia (161).

Physiological modifications occur following recurrent hypoglycemia. In animal models, moderate recurrent hypoglycemia has been shown to have a protective role in preventing age-related decline of hippocampal cognitive function (259) and decreasing brain damage after severe hypoglycemia (260). However, these beneficial effects do not negate the fact that compromised counterregulatory hormone defenses against low blood glucose levels caused by recurrent hypoglycemia persist, which result in psychological and physical morbidities (162). In diabetes, recurrent hypoglycemia decreases glycemic thresholds for counterregulatory responses (203) and decreases neuroendocrine and autonomic responses to subsequent hypoglycemia. In type 1 diabetes, prior, repeated bouts of hypoglycemia cause further suppression of the already impaired counterregulatory hormone responses that are observed with the disease (203;205;220;227;261-263). Following recurrent hypoglycemia, diabetic subjects show markedly diminished epinephrine counterregulation compared with acutely hypoglycemic controls (203;205;223;226;261;263;264). Interestingly, this epinephrine defect was not observed in a study that tested recurrent hypoglycemia with episodes 2 days apart, and the authors suggested that the pathophysiological effect of recurrent hypoglycemia may be of short duration in patients with type 1 diabetes (262) as compared to non-diabetic subjects (265). Since glucagon response is already absent in most diabetic subjects, it has not been shown to be further worsened by recurrent hypoglycemia (203;264). Similar to that in non-diabetic subjects, norepinephrine (205;262;264) and glucocorticoid (205;227;264) responses are not as conclusive since studies have shown either decreased or unchanged counterregulation of these hormones. Recurrent hypoglycemia in type 2 diabetic individuals with moderate
glycemic control has been shown to blunt counterregulation to subsequent hypoglycemia compared non-diabetic controls, and counterregulatory defenses were further impaired in intensively treated type 2 diabetes (219). Glucagon and epinephrine hormone counterregulation, and neurogenic and neuroglycopenic symptom scores to hypoglycemia were reduced following repeated hypoglycemia in type 2 diabetic subjects, and glycemic thresholds were also shifted to a lower glycemic level (168).

Ultimately, hypoglycemia remains a serious complication and limiting factor to glycemic management in both type 1 and type 2 diabetes. Without adequate counterregulation, recovery of blood glucose levels to euglycemia cannot be achieved, and the result is a prolonged state of hypoglycemia with the increased potential for morbidity and mortality.

1.7 Somatostatin
In 1968, rat hypothalamic extracts were demonstrated to inhibit growth hormone secretion from the pituitary gland (266). However, it was not until 1972 that somatostatin, a cyclic tetradecapeptide that inhibited the release of pituitary growth hormone, was first identified and sequenced by the laboratory of Guillemin from sheep hypothalami (267). Guillemin was awarded the Nobel Prize in 1977 for his research on hypothalamic hormones (268). A second form of the peptide with an extension of 14 additional amino acids at the N-terminus, somatostatin-28, was subsequently identified (269). The amino-acid sequences of somatostatin and somatostatin-28 are shown in Figure 1-1. Since its discovery, somatostatin has been found to be widely distributed throughout the body with a broad spectrum of biological effects mediated via a family of five receptors. As such, various analogues for somatostatin designed for therapeutic use began to be synthesized as early
as 1974 (270). Several somatostatin-like peptides have also be identified and reviewed and will be described in brief below.

1.7.1 **Functions**

The best known physiological role of somatostatin is its regulation of hormone secretion, but its other diverse effects include regulation of cell growth, nutrient absorption, smooth muscle contractility, and neuromodulation (271;272). Somatostatin regulates hormone secretion from the pituitary gland and pancreatic islets, gastrointestinal cell function, and immune cell function (271).

1.7.1.1 *General somatostatin effects in pancreatic islets.*

Pancreatic islets have a rich vascular supply and receive up to 10% of the total blood flow to the pancreas despite the fact that they comprise only about 1% of pancreatic tissue (273). Somatostatin’s main effect on pancreatic hormones is inhibition of both insulin and glucagon release (274;275). It also has an autocrine function whereby somatostatin inhibits its own release (272). Pancreatic δ-cells are juxtaposed to insulin- and glucagon-containing β-cells and α-cells, respectively, within islets (276). There is also evidence of paracrine intraislet control exerted by somatostatin since δ-cells are in close proximity to both β- and α-cells in rat and human islets and δ-cell processes were observed to extend into α-cell clusters in rat islets (277;278). Isolated pancreatic islets incubated in a medium containing anti-somatostatin antibodies to bind endogenously secreted somatostatin have significantly increased release of insulin and glucagon (279;280), and isolated pancreata perfused with somatostatin antibody to immnoneutralize islet somatostatin similarly demonstrate increased stimulation of pancreatic glucagon and insulin release (281;282). Furthermore, *in vivo* microscopy experiments of rat and mouse pancreas microcirculation demonstrate a plausible mechanism in which cell-to-cell communication between δ-cells and β-cells can
exist within the islet (283). Taken together, its anatomical juxtaposition to α- and β-cells, findings from in vitro and ex vivo somatostatin immunoneutralization studies, and intra-islet microcirculation experiments all support the concept that somatostatin is a local regulator of pancreatic islet α- and β-cell function.

1.7.1.2 Arguments against local somatostatin effects on pancreatic islets.
Some studies suggest that somatostatin-secreting δ-cells are downstream of glucagon-secreting α-cells and insulin-secreting β-cells in the islet microcirculation of non-diabetic rats, and thus have argued against the regulation of glucagon and insulin by local somatostatin (79;284;285). This hypothesis of islet blood flow implies that pancreatic somatostatin must pass through the systemic circulation before acting on cells of the pancreatic islet. Furthermore, early work with intravenously administered antisomatostatin serum, which has the effect of neutralizing somatostatin’s actions, demonstrated no inhibitory effect on plasma insulin and glucagon levels (286), which contrasts the in vitro and ex vivo effects of antisomatostatin administration and the local role of regulating pancreatic hormone release. However, it should be noted that antisomatostatin antibodies are very large in structure, and if given via an intravenous route, may not be accessible to the interstitium of pancreatic islets for regulation of pancreatic hormone release (88;287).

1.7.1.3 General somatostatin effects in the brain and pituitary gland.
The physiological roles of somatostatin include inhibition of hormone secretion as well as neurotransmission. Somatostatin inhibits GHRH (288) and TRH (289) secretion from the hypothalamus. Within the brain, somatostatin can act as a neurotransmitter with effects on cognition, locomotor, sensory, and autonomic functions (reviewed in: (290-292)). The localization and release of somatostatin from nerve endings supports the transmitter and modulator role of somatostatin (293). Notably, somatostatin also modulates the release of
other brain neurotransmitters: it inhibits the release of norepinephrine, corticotrophin releasing hormone (CRH), and endogenous somatostatin from the hypothalamus – all of which may play a role in the counterregulatory hormone response. Somatostatin inhibits growth hormone release from the anterior pituitary (267;272). Release of thyrotropic hormone, also known as thyroid stimulating hormone (TSH), from the anterior pituitary is inhibited by somatostatin (272). Somatostatin also inhibits the secretion of adrenocorticotropic hormone from the anterior pituitary (ACTH) (271;294), but it has not been shown to affect the reproductive hormones luteinizing hormone (LH), follicle-stimulating hormone (FSH), or prolactin in normal subjects (271).

1.7.1.4 General somatostatin effects in the gastrointestinal tract.

In the gastrointestinal tract, somatostatin acts as a neurotransmitter in the myenteric plexus and as a paracrine regulator in epithelial cells where it influences the function of adjacent cells (272). By and large, somatostatin inhibits gut exocrine secretions of gastric acid, pepsin, bile, and colonic fluid. Somatostatin also suppresses motor activity of the gut by inhibiting gastric emptying, gall bladder contraction, and small intestine segmentation and decreases splanchnic blood flow and glucose absorption from the gut. In general, somatostatin appears to inhibit the majority of gut hormones, such as vasoactive intestinal polypeptide, gastrin, gastric inhibitory peptide, secretin and cholecystokinin (271;295). Somatostatin also inhibits stimulated glucagon-like peptide-1 release from the gut (296;297).

1.7.1.5 Other effects of somatostatin relating to glucose homeostasis.

Somatostatin has well-established effects on regulating the secretion of hormones responsible for maintaining glucose homeostasis. But the question arises whether somatostatin has other direct effects. Somatostatin decreases hepatic glucose output, and
this effect is thought to be secondary to the inhibition of pancreatic hormone secretion (295). In a hyperinsulinemic-euglycemic clamp in non-diabetic humans, somatostatin infusion at a dose commonly used in human investigation (600 $\mu$g/h) did not increase insulin-stimulated glucose uptake in humans (298).

1.7.2 Localization of somatostatin-producing cells

Two main types of somatostatin-producing cells exist and have different morphologies: 1) secretory cells, which have short cytoplasmic extensions; and 2) nerve cells, with multiple branches. In the rat, approximately 65% of total body somatostatin-immunoreactivity is from the gastrointestinal tract, 25% from the brain, 5% in the pancreas, and 5% from other tissues (271).

1.7.2.1 Pancreatic somatostatin-producing cells.

In the pancreas, somatostatin-producing cells are found in $\delta$-cells of the islet. Somatostatin was identified in a population of islet cells that differed from glucagon- and insulin-secreting cells in 1974 (299). In rodent islets, the proportion of $\delta$-cells relative to total cells of the islet (5-10%) is normally far less than that of $\beta$-cells (60-80%) and $\alpha$-cells (15-20%) but greater than that of pancreatic polypeptide (PP)-cells and ghrelin-secreting $\epsilon$-cells (<3%) (300-303). The lower proportion of $\delta$-cells is the same for human islets, in which islet cell composition differs from rodent islets. Numerous recent reports have indicated that compared with rodent islets, the percentage of $\beta$-cells (~50%) and $\alpha$-cells (~40%) is less in humans, but the percentage of $\delta$-cells is similar (~10%) (301;304). Some have suggested that the human islets have as much as twice the proportion of $\delta$-cells as mouse islets (305). This proportion of $\delta$-cells is altered in many models of diabetes, which will be discussed below. Pancreatic $\delta$-cells secrete somatostatin into capillaries or perivascular spaces within islets and normally contribute to approximately 5% of circulating somatostatin (271;306).
Approximately 15% of Δ-cells lack cytoplasmic processes, which implicates a paracrine role for Δ-cells. In the adult, somatostatin-14 is the primary secretory product of pancreatic Δ-cells (307;308). Individual islet cells bind somatostatin-14 differently from somatostatin-28. Although somatostatin-14 binds with α- and β-cells, and somatostatin-28 binds with α-, β-, and Δ-cells, there appears to be a preferential binding association of somatostatin-14 with α-cells and somatostatin-28 with β-cells (309). The differential binding is likely due to the differential distribution of somatostatin receptors on each islet cell type (310), as will be discussed below.

1.7.2.2 Brain somatostatin-producing cells.

The brain is rich with somatostatin-positive neurons and fibers (292;311;312). Somatostatin-cells are abundant in the hypothalamus, brainstem, the deeper layers of the cerebral cortex, and limbic structures. Hypothalamic somatostatin is most well known for its role in inhibiting growth hormone and thyroid-stimulating hormone release from the anterior pituitary (271). Approximately 80% of hypothalamic somatostatin is located in cells of the periventricular nucleus which project their axons to the median eminence, and this comprises the main somatostatinergic tract (271;313). Somatostatin-producing neurons are also located in the paraventricular nucleus (122) which also project to the median eminence (292); arcuate nucleus; supraoptic nucleus; suprachiasmatic nucleus; and ventromedial nucleus of the hypothalamus (293;311;312). In the brainstem region, somatostatin is also localized in the dorsal vagal complex (311;314). Somatostatinergic neurons of the nucleus of the solitary tract project to the hypothalamic paraventricular nucleus (292). As discussed in earlier sections, these hypothalamic nuclei and the dorsal vagal complex are well-known for their role in regulating glucose homeostasis (13;14).
Thus, colocalization of somatostatin in these glucose sensing and regulatory brain regions could implicate a putative role for somatostatin in regulating glucose homeostasis.

1.7.2.3 *Gastrointestinal somatostatin-producing cells.*
Within the gastrointestinal tract, there are two main types of somatostatin-producing cells: D-cells in the mucosa of the pyloric antrum and in the intestine and neurons that are part of submucosa and myenteric plexuses (313;315-317). In the myenteric plexus, somatostatin inhibits the induced release the neurotransmitter acetylcholine (315).

1.7.2.4 *Other somatostatin-producing cells.*
In addition to these main organs that secrete most of the body’s somatostatin, there are other tissue and cell types that also produce somatostatin. Within the thyroid, somatostatin is produced with calcitonin in a subpopulation of C-cells (313;318). Immune and inflammatory cells of the spleen and thymus (e.g. lymphocytes, macrophages) also secrete somatostatin in small amounts when activated (319;320). Somatostatin inhibits the release of growth factors and cytokines and inhibits the proliferation and adhesion of lymphocytes (271;321), presumably as a way of regulating the secretions and functions of these cells. Whereas somatostatin was detected at high levels from extracts of the posterior pituitary, the anterior pituitary had no detectable levels of somatostatin (313;322). There are also small numbers of somatostatin-producing cells in the kidneys and in the adrenal glands (271).

1.7.3 *Synthesis and structure*
Somatostatin is synthesized from the preprosomatostatin gene (323). The rat somatostatin gene encodes preprosomatostatin, a precursor of 116 amino acids that is processed within the endoplasmic reticulum to yield prosomatostatin, a peptide of 92 amino acids (323).
Prosomatostatin is subsequently cleaved posttranslationally to produce somatostatin-28 and somatostatin-14 by prohormone convertases 1 and 2 (PC1/PC2) and carboxypeptidase E (CPE) (272). The two forms of the peptide are found in varying amounts in somatostatin-producing cells due to the differential processing by PC1, PC2, and CPE (271).

Somatostatin-14 can also be derived from alternate processing of somatostatin-28. Somatostatin-14 is the predominant form in the brain, pancreatic islets, retina, peripheral nerves, and enteric neurons. Somatostatin-28 is predominant form in the gastrointestinal tract, especially the duodenum and jejunum, where the prosomatostatin gene is processed in the intestinal mucosal cells. Somatostatin-28 accounts for approximately 20-30% of total immunoreactive somatostatin in the brain (271).

The preprosomatostatin gene in humans has striking homology with other vertebrates. The sequence of somatostatin-14 is completely conserved between fish and mammals, but mammalian somatostatin-28 shares only 40-60% homology with fish somatostatin-28 (324). Two other somatostatin-like peptides have also been identified: cortistatin and neuronostatin.

1.7.3.1 Cortistatin.

A somatostatin-like gene called cortistatin gives rise to two cleavage products which are comparable to somatostatin-14 and somatostatin-28: cortistatin-17 (human) or cortistatin-14 (rat) and cortistatin-29 (human and rat) (325). Cortistatin shares high structural homology with somatostatin and binds to somatostatin receptor subtypes with similar affinity, but its actions, tissue expression patterns, and regulation are not completely the same as that of somatostatin. Cortistatin gene expression was originally thought to be restricted to the cerebral cortex and hippocampus, but its mRNA expression has since been detected in peripheral tissues also known to express somatostatin including the pancreas,
adrenal, liver, and small and large intestine (326). Its presence in the brain is only at a small fraction of the brain with far less distribution (272). Cortistatin has been demonstrated to have some similar hormone regulatory effects as somatostatin (namely, inhibition of growth hormone and insulin release), but it also has additional distinct functions on sleep modulation (327), brain function (325), and effects on immune cells (328).

1.7.3.2 Neuronostatin.

Neuronostatin is a 13-amino acid peptide encoded by the preprosomatostatin gene (329). Its amino acid sequence shares very little similarity to somatostatin (329). It is highly produced in the hypothalamus, and neuronostatin-immunoreactive cells were found to be comparable in number and intensity to somatostatin immunoreactive cells in the hypothalamic periventricular nucleus. However, these peptides differ in their calcium mobilizing effects in cultured rat hypothalamic neurons, which results in diverse cellular activities (somatostatin reduces, while neuronostatin increases, calcium mobilization) (330). For example, intracerebroventricular treatment with neuronostatin increased blood pressure but suppressed food intake and water drinking (329). Furthermore, neuronostatin has no effect on stimulated GH secretion from pituitary. Neuronostatin can also be found in the pancreas where it is thought to be colocalized with somatostatin in islet δ-cells. Importantly, whereas somatostatin activated the Gi-protein signalling pathway mediated via the five different somatostatin receptors, neuronostatin had no such effect, which likely indicates that neuronostatin does not bind to native receptors for somatostatin (329).

1.7.3.3 Apelin

Apelin is a circulating and paracrine peptide hormone which also acts as a neuromodulator. It has various isoforms: apelin-36, apelin-17, and apelin-13 (331). It is secreted by adipose
tissue and is also found in the gastric mucosa and hypothalamus. Specifically in the hypothalamus, apelin is detected in the SON, PVN, and SCN (331). Known for its hypotensive effects (332) and also as an adipokine (333), apelin has more recently garnered attention for its pancreatic effects on insulin inhibition (334;335) and for its localization within the pancreatic islet. Apelin is found in α-cells and β-cells of human, rat, and mouse islets but not δ-cells or PP-cells (335). Apelin itself is upregulated directly by insulin in both human and rodents (336), and islet apelin mRNA expression is suppressed by glucocorticoids (335). Apelin has been shown to lower plasma glucose via increased glucose utilization in fat and muscle (337;338). Similar to somatostatin, the receptor for apelin – the APJ receptor – is a G-protein-coupled receptor which is expressed in various parts of the brain: hippocampus, striatum, thalamus, cortex, cerebellum, and hypothalamus (332;339). The APJ receptor is upregulated in the hypothalamic PVN by acute and repeated stress (340). More recently, APJ expression was found in human and mouse islets (335).

Recently, it was reported that children with type 1 diabetes have increased circulating apelin levels compared to non-diabetic controls. However, no correlations were found between elevated apelin levels and body mass index, glycemia, lipids, and insulin sensitivity in these type 1 diabetic children (341).

1.7.4 Regulation of somatostatin synthesis

The regulatory domains that are located upstream of the 5’ transcriptional unit of the rat somatostatin gene contain: a TATA box, a cAMP response element (CRE), two nonconsensus glucocorticoid response elements (GREs), and a consensus insulin response element CGGA activated by an ETS-related transcription factor (271). Pancreatic duodenal homeobox 1 (PDX-1) is a transcriptional factor required for activating somatostatin gene transcription and maintains pancreatic islet functions along with activating gene transcription of insulin,
islet amyloid polypeptide, glucose transporter type 2, and glucokinase (342). Transcription of the somatostatin gene can be repressed by two silencer elements located in the promoter region (343).

Somatostatin mRNA levels are regulated by many of the factors that also influence somatostatin secretion. Somatostatin gene transcription is stimulated by glucocorticoids, growth hormone, testosterone, estradiol, NMDA receptor agonists, growth factors (IGF-I, IGF-II) and cytokines (IL-1, IL-10, TNF-α, and IFN-γ). Conversely, somatostatin gene transcription is also inhibited by glucocorticoids, insulin, leptin, and TGF-β. Glucocorticoids have a dual effect on the somatostatin gene by enhancing its synthesis via its interaction with the glucocorticoid receptor and GRE and inhibiting its synthesis via accelerated somatostatin transcript degradation. Insulin is also known to accelerate somatostatin mRNA degradation. Among the intracellular mediators known to modulate somatostatin mRNA, Ca²⁺, cGMP, nitric oxide, and cAMP, cAMP most potently activates somatostatin gene transcription. cAMP induces PKA phosphorylation of nuclear cAMP response element binding protein (CREB), which binds to CRE (271).

In neurons, somatostatin gene transcription is strongly activated by binding of the phosphorylated transcription factor cAMP response element-binding protein (CREBP) to the cAMP response element in the promoter sequence of its gene (344;345) and the homeodomain transcription factor PDX-1 to the promoter element TSE₁. In pancreatic islets, somatostatin gene is also upregulated by cAMP and PDX-1. Interestingly, enhancer elements in the promoter region of the somatostatin gene that upregulate its expression in pancreatic islets may actually have the opposite function and act as gene silencer elements in neurons. This includes complexes that bind transcription factors PAX6, PBX, and PREP1 to TSE₂₂ and UE-A elements in the promoter region of the somatostatin gene (272).
Somatostatin has a short half-life (approximately 1.1 to 3 minutes in humans) since it is rapidly metabolized by peptidase enzymes in the blood and tissues. The kidney and liver are the main sites of somatostatin clearance (271).

1.7.5 Regulation of somatostatin secretion

Some agents exert common secretory effects on somatostatin-producing cells throughout the body while others have differing effects depending on the type of tissue. Typically, the divergent effects of these agents occur because of tissue-specific expression of certain receptors for the agent or because of indirect effects through the release of other transmitters or peptides (271). Generally, membrane depolarization of somatostatin-containing nerve cells or peripheral somatostatin secretory cells is a common mechanism that elicits somatostatin release (291).

1.7.5.1 Regulation of pancreatic somatostatin.

Somatostatin release from pancreatic islets δ-cells is normally increased in response to glucose stimulation (305;346) and other nutrients such as amino acids and lipids (271). Glucose stimulation leads to closure of $K_{\text{ATP}}$-channels (presumably via an increase in the cytoplasmic ATP/ADP ratio), and membrane depolarization. Sulfonylureas also stimulate somatostatin release by closing $K_{\text{ATP}}$ channels (347). Glucagon stimulates the release of somatostatin (348). Insulin inhibits pancreatic somatostatin release (271) as does activation of gamma-aminobutyric acid (GABA) receptors on δ-cells (295). Somatostatin secretion by pancreatic δ-cells is stimulated by activation of both β-adrenergic and cholinergic receptors (295). In isolated perfused human pancreas, stimulation of the celiac neural bundle has the predominant effect of inhibiting glucose-stimulated somatostatin secretion via an α-adrenergic mechanism (349). β-adrenergic fibers stimulate somatostatin secretion, whereas cholinergic fibers had a weaker effect (349). Similarly in perfused dog
pancreas, somatostatin secretion was increased by β-adrenergic activation and suppressed by α-adrenergic activation (72). Interestingly, destruction of the VMH increased pancreatic somatostatin release to nutrient stimulation (350). CCKB receptors found on pancreatic δ-cells (351), and more recently, occupation of CCK-1 and CCK-2 receptors was shown to initiate somatostatin secretion in rat pancreatic islet cell line RIN-14B cells (352).

1.7.5.2 Regulation of brain somatostatin.
Unlike its action in the pancreas, glucose inhibits hypothalamic somatostatin secretion, and amino acids do not appear to have a role. Likewise, insulin stimulates hypothalamic somatostatin, which is opposite to its effect in the pancreatic islet. Somatostatin also has an autoregulatory effect whereby it inhibits its own release in the hypothalamus (271). Growth hormone and thyroid hormones enhance somatostatin release, suggesting that somatostatin plays a negative feedback role on regulating these hormones (353;354). Secretion of somatostatin from the hypothalamus is promoted by dopamine, substance P, glucagon, acetylcholine, norepinephrine, vasoactive intestinal polypeptide, and cholecystokinin (318;355). Various cytokines, such as IL-1, IL-6, and TNF-α, also stimulate somatostatin secretion from the rat brain (356). In contrast, leptin and TGF-β have been shown to inhibit somatostatin secretion from rat hypothalamic cells (357;358). Opiates and GABA likewise inhibit somatostatin release.

1.7.5.3 Regulation of gastrointestinal somatostatin.
Although the focus of this thesis is not on gastrointestinal somatostatin regulation, it is important to briefly mention stimulators and inhibitors of somatostatin from the gut since it represents the main source of circulating somatostatin (271). Gut secretion of somatostatin is triggered by luminal, but not circulating somatostatin (291;359). Stimulation of the vagus nerve induced secretion of somatostatin into the antral lumen (360). Somatostatin
release is also stimulated by insulin (271) as well as GLP-1, but not GLP-2 (361). Gastrin-releasing peptide stimulates somatostatin secretion from the intestine, but galanin, vasoactive intestinal peptide, and epinephrine had no effect (361). Bombesin, a neuropeptide and hormone that stimulates gastric release and aids in negative feedback signals to suppress eating, also stimulates somatostatin (362). In the fasting state, plasma concentration of somatostatin is low (30–100 pg/mL) and increases by approximately 2-fold in the postprandial state (363). This postprandial increase in plasma somatostatin is mostly due to somatostatin derived from the intestinal epithelium (364;365).

### 1.8 Somatostatin receptors (SSTRs)

Somatostatin receptors (SSTRs) were first described in 1978 in pituitary cell lines (366), but it was not until 20 years after the discovery of somatostatin that structure of one of its receptors was characterized by molecular cloning (367). It is now known that SSTRs are found in various densities in the brain, pituitary gland, gastrointestinal tract, endocrine and exocrine pancreas, adrenal glands, thyroid, kidneys, and immune cells (271). Tumour cell lines are also rich in SSTRs, but a review of this literature is beyond the scope of the present thesis. For the purposes of this thesis, the general localization and function of the family of receptors for somatostatin, SSTR1 to SSTR5, inclusive, will be briefly examined, and more emphasis will be taken to examine our SSTR of interest, SSTR2.

Somatostatin binds to each of the SSTRs with very high affinity; that is, in the nanomolar range (368). Somatostatin-14 binds to all the SSTRs with greater affinity with the exception of SSTR5, in which somatostatin-28 is the ligand with greater selectivity (369). The receptors can also be grouped into two subfamilies in which SSTR2, 3, and 5 bind well
to short somatostatin analogues (i.e. hexapeptide and octapeptides) and SSTR1 and 4 which have poor binding with these short peptide analogues (369). In general, the SSTR subtypes are of a similar size (356-391 amino acids in length), and each has seven $\alpha$-helical transmembrane segments typical of G-protein coupled receptors with an extracellular N-terminus and an intracellular C-terminus. In the seventh transmembrane segment, there is a signature sequence for this family of receptors that is highly conserved in all SSTR isoforms from humans and other species. The five SSTR subtypes display a high level of structural conservation across species (369;370). The nearest related family of receptors to SSTRs are the opioid receptors (371).

1.8.1 General localization of SSTRs
The central nervous system, pituitary, and gastrointestinal tract ubiquitously express all members of the SSTR family, SSTR1-5. In the central nervous system, SSTR1 is most highly expressed in the neuraxis (the axis of the central nervous system) and throughout the entire brain. SSTR2 has a high level of expression in the cerebral cortex. SSTR3 has preferential expression in the cerebellum, SSTR4 is less well expressed as compared to the other types and in early studies was referred to as the “brain-specific” SSTR (372), and SSTR5 is moderately well expressed throughout the brain (373-377). Interestingly, brain SSTR5 in humans is less well expressed than in rats (376). Specifically in the hypothalamus, SSTR1 has the highest overall expression, followed by SSTR2, 4, 3 and 5. SSTR1 seems to be highest expressed in neurons of all major hypothalamic nuclei, in nerve fibers along the median eminence, and along the third ventricle. SSTR2 is likewise expressed in these areas but at a lower density than SSTR1. SSTR3 is localized in the paraventricular nucleus, dorsomedial nucleus, arcuate nucleus, and median eminence. SSTR4 is localized to the arcuate nucleus, ventromedial nucleus, and median eminence.
SSTR5 is the least expressed subtype and appears in the median eminence (374;375;378).

In the pituitary, all five subtypes of SSTR are expressed in rats, but human pituitaries lack SSTR4. SSTR5 is the most predominant subtype expressed in the pituitary, followed by SSTR2 (373;379-382). In the gastrointestinal tract, all five SSTRs have been identified in the stomach and small and large intestine, and the enteric plexus expresses SSTR1-3 (383;384).

Rat and human pancreatic islets also express all five SSTR subtypes, but the species differ in their level of SSTR expression on the different islet cell types. In human pancreatic islets, insulin-secreting β-cells highly express SSTR1 and SSTR5, glucagon-secreting α-cells predominantly express SSTR2, and somatostatin-secreting δ-cells predominantly express SSTR5 (385). In human isolated islets, it was demonstrated that SSTR2 was the predominant subtype that mediates the suppression of glucagon release by somatostatin (386). In rodent islets, the main difference is that β-cells more exclusively express SSTR5 (387). Rat α-cells and δ-cells show the same selective predominant expression of SSTR2 and SSTR5, respectively (388-391). Functional studies in rodents are consistent with the above findings of receptor localization showing that SSTR2 mediates the inhibitory effect of somatostatin on glucagon secretion while SSTR5 inhibits insulin release (388;389;392-394). Notably, one group has reported slightly different findings in which rat and mouse α-cells showed high expression of SSTR2 and SSTR5 and β- and δ-cells showed high levels of SSTR1, 2 and 5, suggesting that more than one SSTR needs to be activated to suppress islet glucagon and insulin release (395;396).

In the adrenal gland, there is a high concentration of SSTR2 (390;397-399) and also moderate levels of SSTR1 and SSTR3 (373;400). In the liver and kidney, SSTR3 is expressed. The lung, heart, and placenta express SSTR4. Immune cells preferentially
express SSTR2 (319;401;402), but recent studies report that SSTR1 and SSTR2 colocalized on human macrophages are responsible for the immunosuppressive effect of somatostatin (403). The retina expresses all SSTRs, with highest levels of SSTR1 and SSTR2 (404). Interestingly, the majority of human tumour cells are usually positive for SSTRs (271) with predominant highest frequencies reported for SSTR2 (405) or SSTR3 mRNA expression (406-408). More specifically in human neuroendocrine tumours, a recent study demonstrated that SSTR1 and SSTR5 were the most highly detected forms of receptors, followed by SSTR3 and SSTR2 (409).

1.8.2 **General functions of SSTRs**

SSTRs mediate the functions of somatostatin. As previously mentioned, one of the main functions of somatostatin is to inhibit exocytosis in target cells. This action is mediated predominantly via SSTR2, SSTR3, and SSTR5 and involves i) decreased intracellular cAMP due to inhibition of adenylyl cyclase, ii) decreased Ca\(^{2+}\) influx due to action on K\(^+\) and Ca\(^{2+}\) ion channels, and iii) stimulation of phosphatases such as calcineurin (which inhibits exocytosis) and serine threonine phosphatases (which dephosphorylate and activate K\(^+\) and inhibit Ca\(^{2+}\) ion channels) (369). In addition to its anti-secretory effects, somatostatin also has: 1) anti-proliferative, 2) anti-growth, and 3) apoptotic functions (271). The anti-proliferative effects of somatostatin are mediated primarily via SSTR1, 2, 4, and 5 by which it induces cell cycle arrest at the G1 phase. Anti-proliferative effects of somatostatin have been demonstrated in normally dividing cell types such as activated lymphocytes and inflammatory cells (410;411), intestinal mucosal cells (318), bone and cartilage cells (412), as well as tumour cells (271). SSTRs activate phosphotyrosine phosphatases (PTPs) to transduce anti-growth signals via modulation of a mitogen-activated protein kinase (MAPK) pathway. SHP-1 and SHP-2 belong to the family of PTPs which are implicated in

1.8.3 Specific localization, ligand binding, and signalling of SSTR2

The rat SSTR2 gene was identified in 1992 (416). Two spliced variants of the SSTR2 gene exist: a long form (SSTR2A), and a short form (SSTR2B). These two variants are generated through alternate mRNA splicing and differ only in the length of their cytoplasmic tail, with the SSTR2B form differing in 15 amino acids at the C-terminus (400;417). The C-terminal tail is not essential for coupling to G-proteins or adenylyl cyclase. There is a 93-96% conservation of the sequence identity of SSTR2 isoforms among human, rat, mouse, porcine, and bovine species.

SSTR2 is highly prevalent in the kidney, and pancreas and along with SSTR1 is prevalent in the stomach, pituitary, small intestine (particularly the duodenum), and spleen (271;367;385). It is the main subtype expressed in lymphocytes and inflammatory cells (319;401;402). There are also low levels of SSTR2 detected in the liver (367) although in healthy liver, SSTR2 may be localized on other cell types such as cholangiocytes rather than hepatocytes (418). SSTR2 is the main subtype responsible for the suppression of gastric acid (388).
In human pancreatic islets, SSTR2 is predominantly expressed on glucagon-secreting α-cells (385). In human isolated islets, it was demonstrated that SSTR2 was the predominant subtype that mediates the suppression of glucagon release by somatostatin (386). Rat α-cells show the same selective predominant expression of SSTR2 (388-391). Functional studies in rodents are consistent with the above findings of receptor localization showing that SSTR2 mediates the inhibitory effect of somatostatin on glucagon secretion (388;389;392-394).

In the brain, moderate to high levels of SSTR2 expression include the basolateral amygdala (BLA) (419-421); in neurons of all major hypothalamic nuclei (378;422), including mediobasal hypothalamus (312;423) and specific nuclei regulating endocrine and fluid homeostasis such as the supraoptic nucleus (SON) and PVN and feeding centers such as the ARC and dorsomedial nucleus (420;424-428); cerebral cortex (419;420); parabrachial nucleus, locus coeruleus, and NTS of the brainstem (419;420), and in the nerve fibers along the median eminence (378). Intracerebroventricular administration of a selective SSTR2 agonist elicited neuronal activation (as measured by an increase in Fos expression) in brain regions such as the BLA, SON, PVN, ARC, parasubthalamic nucleus, lateral parabrachial nucleus (429). A recent study reports that selective activation of brain SSTR2 induces a feeding response while blocking brain SSTR2 reduced food intake (430). Interestingly, the effect of this antagonist was specific to central administration and not reproducible by intraperitoneal administration. Drinking, grooming, and locomotor activity were also increased by selective activation of brain SSTR2 (430).

With SSTR5, SSTR2 inhibits growth hormone and thyroid stimulating hormone from human and rat pituitary cells (431;432). However, there is also evidence demonstrating the importance of SSTR1 in regulating GHRH and growth hormone release (423;428;433;434).
SSTR2 is not internalized as readily as the other SSTRs (435-437). From studies that demonstrated that SSTR2 is sensitive to GTP analogues and pertussis toxin, it is now known that SSTR2 is associated with inhibitory G-proteins $G_i$ and $G_o$, which are the G-proteins that involved with the inhibition of adenylyl cyclase (438).

1.9 Somatostatin in type 1 diabetic humans and animal models

Somatostatin levels are increased in many diabetic subjects studied. The most notable change is the increase in pancreatic somatostatin content which is has been observed in type 1 diabetic humans (439) in which an increased number and volume of $\delta$-cells has been observed (440). Pancreatic somatostatin release was elevated in type 1 diabetic subjects (363;441), particularly in those with poor glycemic control (441). Increased basal plasma somatostatin has also been reported in type 1 diabetic individuals (442;443). Experimental diabetes induced in monkeys (444) showed increased $\delta$-cell volume and hyperplasia. Alloxan-diabetic dogs also demonstrated elevations in islet somatostatin content and $\delta$-cell area (90;445) as well as increased basal and nutrient-induced circulating somatostatin levels (446) as compared to non-diabetic dogs.

There is an abundance of literature demonstrating increased somatostatin levels in streptozotocin- (STZ-) diabetic rats. STZ-diabetes is an experimental model of diabetes (discussed in section 3.1.2). STZ-diabetic rats have increased pancreatic somatostatin (174;439;447-452) and secrete more pancreatic somatostatin than non-diabetic controls (451). It was demonstrated that pancreata from STZ-diabetic rats had more pancreatic somatostatin content which increased in proportion to the dose of STZ used to induce diabetes while graded reductions of insulin content were observed (453). In addition, these
pancreata had increased arginine-stimulated secretion of somatostatin, which also negatively correlated with insulin release. This suggests that pancreatic somatostatin content and secretion are proportional to the state of insulin deficiency (453). Pancreatic prosomatostatin mRNA was increased in STZ-diabetic rats compared to non-diabetic rats (205;454). Plasma somatostatin levels were also elevated in STZ-diabetic rats (174;205;455). Fasting reduced basal plasma somatostatin in both STZ-diabetic and non-diabetic rats (455). The stomach of STZ-diabetic rats was also shown to have elevated levels of somatostatin as compared to non-diabetic rats (452;456;457). These D-cells of STZ-diabetic rats also secreted more somatostatin when stimulated than those of non-diabetic counterparts (456). Small intestine somatostatin content was also increased in STZ-diabetic rats compared with control animals (457). Intravenous arginine increased plasma somatostatin levels to a greater extent in STZ-diabetic rats as compared to non-diabetic rats (455).

Alloxan-diabetes is another form of induced, experimental diabetes. In alloxan-diabetic rats, pancreatic δ-cell mass was increased (458;459), as was arginine-stimulated somatostatin release (459). In one study, hyperplasia of δ-cells was observed after 14 months of alloxan diabetes (458).

Increased pancreatic somatostatin has also been reported in NOD mice (460). In NOD mice 15 days after onset of diabetes, pancreatic islets were characterized by infiltration of lymphocytes, and somatostatin-containing cells occupied the majority of endocrine cells of the islets while insulin-containing cells decreased as expected and glucagon-containing cells remained similar (460).
There is evidence in animal models of diabetes that insulin treatment can reduce and/or partially normalize pancreatic somatostatin levels (90;351;452), arginine-induced elevations of plasma somatostatin (455), and high basal somatostatin secretion rates in islets from untreated diabetic rats (461). This suggests that increased somatostatin during diabetes may be due, at least in part, to a lack of endogenous insulin action or its metabolic consequences. However, it should be noted that insulin treatment of type 1 diabetic humans did not prevent the abundance of δ-cells found in human diabetic pancreata (439) nor the elevation in plasma immunoreactive somatostatin levels (363). In addition, mRNA expression of pancreatic prohormone convertases PC1 and PC2 were found to be >2-fold and >4-fold increased, respectively, in STZ-diabetic rats, which may also be in part responsible for increased pancreatic somatostatin in diabetes (462). It has also been postulated that increased glucagon, representing an absolute or relative glucagon excess, within the pancreatic islet might lead to a compensatory response of the δ-cell to secrete more somatostatin. This postulate was supported by observations that glucagon-secreting α-cell tumours also showed hyperplasia of δ-cells (439).

Whereas glucose stimulates somatostatin-secretion from healthy δ-cells, somatostatin-secreting δ-cells from islets from STZ-diabetic rats were unresponsive to changes in glucose concentrations but still responded to increased cAMP levels (461). This suggests that the hyperglycemia associated with diabetes may not be responsible for the increased pancreatic somatostatin release observed in diabetes. *In vivo* insulin treatment of STZ-diabetic rats restored the glucose sensitivity of δ-cells (461). Literature also widely supports the concept that somatostatin release is stimulated by glucose and tolbutamide in healthy individuals, but that these effects are lost in type 1 and type 2 diabetes, which has been proposed to
contribute to the impaired prandial suppression of glucagon secretion in diabetes (305;463-468).

1.9.1 Brain somatostatin in experimental diabetes

The literature regarding brain somatostatin levels is less consistent than that of the pancreas and gastrointestinal tract. In untreated STZ-diabetic rats, somatostatin mRNA expression was reduced in the central portion of the periventricular nucleus with no changes observed in the other somatostatin-rich hypothalamic areas: the periventricular nucleus or the arcuate, suprachiasmatic or paraventricular nuclei (469). Interestingly, this anatomical area of somatostatin reduction is involved with regulation of the anterior pituitary hormone release. These changes in hypothalamic somatostatin expression were restored with insulin treatment that normalized blood glucose levels (469). Older studies, however, did not observe changes in hypothalamic somatostatin content or mRNA expression in STZ-diabetic as compared to non-diabetic rats (448;449;452;470). Similarly, there was no change in hypothalamic somatostatin content of db/db and ob/ob mice (471). It is possible that no changes were observed if total hypothalamic content were measured as opposed to specific anatomical areas. Interestingly, others have observed increased hypothalamic secretion of somatostatin into the hypophyseal portal blood in vivo in STZ-diabetic rats (472). In other areas of the brain, decreased somatostatin mRNA expression in the hippocampus and frontal cortex of STZ-diabetic rats has also been reported (473), but how and whether these anatomical changes affect glucose homeostasis in diabetes has yet to be determined.
1.10 Somatostatin in type 2 diabetic humans and animal models

Reports of somatostatin levels and δ-cell function in type 2 diabetes are not as consistent as that described in type 1 diabetes. Pancreatic somatostatin secretion was shown to be greater in type 2 diabetic humans, to a similar extent as type 1 diabetic subjects, as compared to non-diabetic individuals (441). The number of somatostatin-expressing δ-cell was increased by 58% in type 2 diabetic human pancreases as compared with non-diabetic human pancreases and represented an absolute increase in number (474). However, other studies showed no difference in percentage volume (475) or number (440) of δ-cells in type 2 diabetic human pancreatic islets or in basal plasma somatostatin levels (476).

In db/db mice, increased pancreatic somatostatin content and δ-cell hyperplasia were observed. In ob/ob mice, pancreatic somatostatin content was also increased, but no increase was noted in the number δ-cells (471). However, other studies in db/db and ob/ob mice demonstrated that there were no differences in hypothalamic, gastric, or pancreatic somatostatin levels in these genetically diabetic and/or obese mice until their serum insulin levels decreased (477). Based on the temporal relationships of pancreatic somatostatin to the disturbances in carbohydrate metabolism, the authors suggest the concept that changes in pancreatic somatostatin represent a response to, rather than a cause of, hypoinsulinemia (477).

1.11 Diabetes-induced changes in SSTR expression

Some of the studies that investigated changes in SSTR levels in diabetic animal models had the purpose of examining growth hormone regulation and thus focused their investigations on the brain and pituitary gland. Some older studies did not discern between which subtype
of SSTR was detected. In STZ-diabetes of short duration (5 and 9 days of diabetes), concentrations of SSTRs in the hypothalamus and anterior pituitary were greatly reduced and were not corrected by insulin treatment (478). More specifically, one group showed that hypothalamic mRNA expression of SSTR5, but not SSTR1-4, and pituitary mRNA expression of SSTR1, 2, 3, and 5, but not SSTR4, was reduced in STZ-diabetic rats (479). In these animals, insulin treatment restored hypothalamic SSTR5 levels and partially restored pituitary SSTR1 and SSTR5 levels. It was suggested that chronic exposure to high somatostatin levels in diabetes may account for reduced SSTR mRNA expression due to receptor desensitization, and insulin, high blood glucose levels, or glucocorticoids may also in part regulate the expression of hypothalamic and pituitary SSTR mRNA expression (479). However, the explanation behind the subtype-specific reduction is not yet apparent. SSTRs in the cerebral cortex were not affected by STZ-diabetes of short duration (478).

### 1.12 Somatostatin: hypoglycemia-induced changes

Circulating somatostatin levels increase in response to insulin-induced hypoglycemia. This has been observed both in non-diabetic and type 1 diabetic patients without diabetic autonomic neuropathy (236;480). However, plasma somatostatin levels are not elevated in response to hypoglycemia in subjects with diabetic autonomic neuropathy, thus underscoring the importance of autonomic nervous system activity in pancreatic hormone regulation during hypoglycemia (480). The increase in circulating somatostatin to insulin hypoglycemia is mediated via activation of the vagus nerve and is in part regulated by the associated increase in gastric acid (481;482). Truncal vagotomy also abolished the rise in somatostatin to insulin-induced hypoglycemia in humans. It was concluded that the somatostatin response is dependent upon vagal integrity and that section of the vagus
nerve unmasks a suppressive effect of insulin action or its metabolic or hormonal consequences on circulating somatostatin levels (483). There is also evidence that activation of the \( \beta \)-adrenergic system by hypoglycemia may stimulate increased secretion of somatostatin (451;484). Under low glucose conditions, locally secreted glucagon was shown to increase somatostatin secretion in isolated islets via L-glutamate, a co-secretory product from \( \alpha \)-cells that acts on a glutamate receptor on \( \delta \)-cells, at least in islets on non-diabetic rats (485). This response of somatostatin to hypoglycemia is not dependent on residual \( \beta \)-cell function since this rise in somatostatin was demonstrated in both type 1 diabetic patients with and with residual \( \beta \)-cell function (486). This rise in plasma somatostatin was similar in magnitude in both non-diabetic and diabetic individuals (486). Although insulin-induced hypoglycemia elicited an increase in plasma somatostatin, there was no rise of somatostatin to surgical stress, thus it has been suggested that this elevation of somatostatin is likely specific to metabolic stress in non-diabetic humans (487).

Circulating somatostatin levels increase with insulin-induced hypoglycemia but not with insulin infusion with dextrose to maintain euglycemia, thus this rise in plasma somatostatin is not due to the effect of insulin but is stimulated indirectly via hypoglycemia (488). In type 2 diabetic individuals, no rise in plasma somatostatin was observed in response to insulin-induced hypoglycemia although an increase was observed in non-diabetic individuals with equivalent hypoglycemia (476).

It is important to note that one of the problems in interpreting plasma somatostatin responses is the origin of the circulating somatostatin since it can originate from various tissues (318;489), especially since stimuli such as insulin, glucose, and hypoglycemia can have divergent effects on hypothalamic and pancreatic somatostatin release, as mentioned in an earlier section.
1.12.1 Hypoglycemia-induced somatostatin and SSTR changes in the brain

Interestingly, hypothalamic somatostatin mRNA expression levels were reported to be dramatically increased by insulin-induced hypoglycemia in vivo (490). In numerous in vitro studies, both low glucose concentrations and intracellular glycopenia induced with 2-deoxy-D-glucose markedly stimulated somatostatin release from rat hypothalamic fragments (491-494). Glucopenia induced by 2-deoxy-D-glucose, however, did not alter hypothalamic somatostatin gene expression (494).

In contrast, it has been demonstrated that severe hypoglycemia of a longer duration than the studies described above produces a disappearance of somatostatin-like immunoreactivity in the dorsal hippocampus and frontal cortex (495). Furthermore, other earlier studies demonstrated that hypoglycemia did not affect somatostatin levels in the hypothalamus, cerebral cortex, hippocampus, and striatum (496-498). However, the total number of SSTRs (subtype not specified) decreased in the hippocampus and striatum in response to insulin-induced hypoglycemia as compared to controls but was unchanged in the hypothalamus and cerebral cortex (496;497).

1.13 Somatostatin and its analogues in diabetic therapy

Type 1 and type 2 diabetic individuals have a decreased secretory response of somatostatin to glucose stimulation, which has been postulated to contribute to impaired prandial glucagon inhibition (305;463-468), and hyperglucagonemia can stimulate hepatic glucose output, thus worsening diabetic hyperglycemia (463;464;467). Thus, somatostatin has also been considered as an adjunct therapy with insulin for the treatment of diabetes (489;499;500). The mechanism for improved glycemic control with somatostatin was
based on the somatostatin’s action to suppress glucagon and growth hormone secretion and delay carbohydrate absorption (489). Due to the short half-life of the somatostatin peptide, synthetic analogs, such as octreotide, were synthesized (501-503). However, somatostatin analogues have not consistently been shown to be a beneficial adjunct therapy for diabetes (504;505).

1.14 Somatostatin antagonist – PRL-2903

Functional biochemistry studies have shown that four amino acids (Phe-Trp-Lys-Thr) in the native somatostatin peptide form a β-turn that is essential for the biological activity of somatostatin (506;507). With this knowledge, various agonists and antagonists with greater metabolic stability and/or greater binding affinities have been synthesized with pharmacological selectivity for particular SSTRs.

The SSTR2 peptide antagonist used in the present thesis is PRL-2903 and has the chemical structure H-Fpa-cyclo[DCys-Pal-DTrp-Lys-Tle-Cys]-Nal-NH₂ (Figure 1-1, Figure 1-2). This peptide was synthesized by Dr. David Coy at Tulane University, Head of Peptide Research Labs (New Orleans, LA, USA) (508;509). It is also referred to as DC-41-33 and BIM-23458. This octapeptide has a molecular weight of 1160 da. The peptide has an IC₅₀ of 2.5 nM in a rat pituitary growth hormone in vitro antagonist assay versus somatostatin (1 nM) and it binds to cloned human SSTR2 with a $K_i$ of 26 nM. 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 (509).
**Figure 1-1.** Linear structures of somatostatin, somatostatin-28, and SSTR2 antagonist PRL-2903.

**Figure 1-2.** Structure of SSTR2 antagonist PRL-2903.
2 General Objectives

Hypoglycemia can have a profound impact on the lives of individuals with diabetes, and it this population may fear hypoglycemia more than the long-term diabetic complications (1;258). Thus, minimizing the incidence of hypoglycemia will improve the quality of life of people with diabetes until a cure or prevention of diabetes is found.

The main objective of this thesis was to determine a means to improve the counterregulatory response to hypoglycemia – in particular the glucagon response, which plays a key role in promoting glycemic recovery (510), and which has consistently been reported to be impaired in diabetes (167;168;511-513).

2.1 Specific Aim: Study 1

As described in the Introduction and will be further discussed in the chapters ahead, it is recognized that the impairment in the glucagon response to hypoglycemia in diabetes can be due to several factors. In brief, these mechanisms are related to local effects in the pancreatic islet (76), or to central effects in brain regions known to detect glucose levels and activate glucoregulatory responses (13;193). In addition to these reported factors, we hypothesized that in diabetes during hypoglycemia, glucagon is suppressed by the prevailing inhibitory effect of excessive somatostatin acting via SSTR2. Somatostatin has
inhibitory effects on the stimulated release of glucagon in the pancreas via SSTR2. Furthermore, somatostatin and SSTR2 are found in brain regions that regulate glucose-sensing and glucoregulatory response, and somatostatin is also known to have inhibitory actions on the secretion of neuropeptides that are involved with the activation of counterregulatory hormone responses to hypoglycemia. Thus, the first objective of this thesis was to determine whether defective glucagon, and other hormone, counterregulation can be restored in diabetic rats if somatostatin actions are inhibited by using a selective SSTR2 antagonist.

2.2 Specific Aim: Study 2

In light of the fact that we were able to demonstrate a restoration of the glucagon response, and markedly improved corticosterone response, to hypoglycemia in diabetic rats, we then endeavoured to determine whether such hormone improvements could translate to enhanced recovery of hypoglycemia, or prevention of hypoglycemia. From our first study, we demonstrated deficient glucagon and corticosterone counterregulation to hypoglycemia in diabetic rats. However, in this model of diabetes, the epinephrine response was not impaired. Thus, we wanted to test our hypothesis of counterregulatory improvement in a model with a well-known epinephrine counterregulation defect in addition to impairments of glucagon and corticosterone – recurrent hypoglycemia (203;205;223;226;261;263;264). We hypothesized that blocking the actions of somatostatin via SSTR2 would promote glycemic recovery. Thus, the second objective of this thesis was to determine whether counterregulatory responses can also be restored by SSTR2 antagonist in diabetic rats exposed to recurrent hypoglycemia and if such improvements can lessen the severity of subsequent hypoglycemia.
Chapter 3
General Materials and Methods

3  General Materials and Methods

3.1  Experimental animals

3.1.1  Care and maintenance

Male Sprague-Dawley rats (Charles River Laboratories, Saint-Constant, QC, Canada) with an initial body weight of 275-300 g were used. Rats were individually housed in opaque cages in temperature (22-23°C) and light-controlled (12-h light:12-h dark cycle; lights on between 0700 and 1900) rooms. Rats had free access to drinking water and fed ad libitum with rat chow (Teklad Global 18% protein, Harlan Laboratories, Madison, WI, USA). The rats were allowed 1 week to acclimatize to experimenter handling and their environment before experimental manipulation. Blood glucose, body weight, and food intake were monitored for a minimum of 5 days per week for the duration of the experiment. Morning (fed state) blood glucose was measured using a handheld glucometer (Ascencia Elite handheld glucometer, Bayer Canada Ltd., Etobicoke, ON, Canada) via a tail-nick blood sample. 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.

3.1.2  Streptozotocin-diabetic rat model of diabetes mellitus

Streptozotocin (STZ) consists of a 2-deoxy-ß-glucose molecule substituted at C2 with a N-methyl-N-nitrosourea group (514). The structure of STZ is shown in Figure 3-1 (514). STZ is a diabetogenic agent used to induce experimental diabetes in animals via its cytotoxic
effects on pancreatic β-cells (515). The deoxyglucose moiety of STZ directs it to the pancreatic β-cell where it is taken up via glucose transporter GLUT2 (516). The expression of GLUT2 on β-cells is needed for the diabetogenic action of STZ (517). The nitrosurea moiety of STZ confers its cytotoxic effect.

![Figure 3-1. Structure of streptozotocin; see text for reference.](image)

It is suggested that β-cell death is the result of one or more of the following mechanisms: 1) DNA alkylation; 2) nitric oxide (NO formation); 3) free radical formation. It is proposed that the primary mechanism for STZ-induced β-cell death is DNA fragmentation resulting from DNA alkylation, which related to the nitrosurea moiety of STZ (518). DNA is alkylated by highly reactive carbonium ions (CH₃⁺) are formed by the decomposition of nitrosurea (519). NO is liberated when STZ is metabolized and is known to have cytotoxic effects on β-cells (520). NO also partially mediates a restriction of mitochondrial ATP generation (516). The reduction of ATP generation results in high intracellular levels of ADP which causes mitochondrial damage and the generation of hypoxanthine. Xanthine oxidase (XOD), the activity of which is high in β-cells, catalyzes the oxidation of hypoxanthine to xanthine and xanthine to uric acid, both steps yield hydrogen peroxide and free oxygen radicals as byproducts. Since β-cells are deficient in superoxide dismutase, they are rendered inefficient at scavenging free radicals and are thus more susceptible to the effects of STZ (519). Along with DNA alkylation, the production of NO and reactive oxygen species further contribute to DNA damage, which ultimately leads to an excision and repair process by poly
(ADP-ribose) synthase. This leads to the depletion of cellular nicotinamide adenine dinucleotide (NAD\(^+\)), a substrate for poly (ADP-ribosylation) and further reduction of cellular ATP, which results in the subsequent inhibition of insulin synthesis and secretion (521).

To summarize, the toxicity of STZ is especially relevant to \(\beta\)-cells due to several intrinsic properties of \(\beta\)-cells: 1) intrinsically low levels of protective superoxide dismutase; 2) intrinsically low content of the substrate NAD\(^+\), which is needed for DNA repair; 3) intrinsically high activity of XOD, which forms free radicals; and 4) intrinsic expression of GLUT2, which is the transporter by which STZ enters the \(\beta\)-cell. Thus, \(\beta\)-cells are extremely vulnerable to STZ-toxicity. STZ-induced experimental diabetes is sometimes criticized for its non-\(\beta\)-cell effects. Some have argued that other GLUT2-expressing tissues, such as hypothalamic astrocytes (522), may be compromised by STZ. Nevertheless, the administration of STZ in rats generates metabolic and endocrine changes that are relevant for the present study and remains a good a model for diabetes. Approximately 70% of \(\beta\)-cells are destroyed with a single, moderate dose of STZ (65 mg/kg) (470), and thus in contrast to fully developed type 1 diabetes in which endogenous insulin secretion is completely absent (523), this form of diabetes is less severe. STZ at 65 mg/kg yields a diabetic model characterized by fasting and fed hyperglycemia, normal fasting plasma insulin levels, but reduced fed-state plasma insulin levels (524).

3.1.3 **Induction of diabetes using streptozotocin**

Streptozotocin (STZ, 65 mg/kg, Sigma Chemical Co., St. Louis, MO, USA) was dissolved in 0.9% saline and immediately injected intraperitoneally to induce diabetes in the rats.

Within 24 h of STZ injection, post-prandial plasma glucose levels were elevated, food intake was increased, and body weight was less than that of saline-injected controls. Rats that did
not exhibit hyperglycemia (i.e. > 10 mM) 48 h following injection of STZ were excluded from the study.

3.1.4 Chronic vessel catheterization

Chronic, indwelling catheters were implanted in the left carotid artery and right jugular bein to allow for undisturbed blood sampling and re-infusion of red blood cells. Surgery was performed in designated operating rooms at the University of Toronto’s Department of Comparative Medicine animal facility under aseptic conditions. 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.

3.1.4.1 Pre-operative preparation.

Surgical instruments, gauze, cloths, and gloves were autoclaved prior to use. Sterile, new syringes, needles, and sutures were used. Catheters were made from polyethylene tubing (PE-50, length: 80 cm, ID: 0.58 mm, OD: 0.965 mm, Becton Dickinson, Sparks, MD, USA) with a 3.0 cm- overlap of silastic tubing (ID: 0.76 mm, OD: 1.65, Dow Corning Corporation, Midland, MI, USA). The silastic overlap was dipped in diethylether to expand the tubing prior to being slipped over the end of the polyethylene catheter, forming an overlap of 1.5 cm. The silastic end of the catheter was then bevelled at 45° to facilitate insertion into the vessel. Catheters were soaked in 70% ethanol to disinfect for at least 1 hour, flushed with saline, and primed with 10 USP/mL heparinized saline (Heparin LEO; 1000 USP units/mL; LEO Pharma Inc., Thornhill, ON, Canada) prior to vascular insertion.

3.1.4.2 Surgical procedure.

Rats were anesthetized using a ketamine cocktail, which consisted of ketamine (100 mg/kg, ip; MTC Pharmaceuticals, Cambridge, ON, Canada), acepromazine (1 mg/kg, ip; Wyeth-
Ayerst Canada Inc., Montreal, QC, Canada), and xylazine (1 mg/kg, ip; Bayer Inc., Etobicoke, ON, Canada). Once rats were anesthetized, the ventral side of the neck and the dorsal side of the neck between the scapulae were shaved and cleaned with 70% ethanol and 10% povidone-iodine (Betadine solution, Purdue Pharma, Pickering, ON, Canada). The rat was placed in the dorsal recumbent position on an operating tray, and its chin and limbs were fastened to the tray with masking tape. The rat was covered with sterile surgical cloths exposing only the incision area. The ventral skin just anterior to the collarbone was cut with a 2-cm transverse incision. Using blunt dissection through muscle layers, the left carotid artery was isolated from the vagus nerve and connective tissue. The exposed vessel was ligated at the cranial end using sterile 3-0 silk thread. Another ligature was loosely tied at the caudal end of the exposed carotid. To temporarily stop blood flow to this segment of the vessel, the exposed carotid artery was pulled taut at the 2 ligatures. Scissors were used to make a small incision in the vessel wall. The primed, bevelled silastic end of the catheter was inserted through the opening in the carotid, past the loose caudal-end ligature, until the silastic-polyethylene overlap of the catheter was also inside the vessel. The catheter was then secured by tightening the loose ligature at the caudal end. Two additional sutures were tied to anchor the catheter to the vessel. The catheter was then tested to ensure blood withdrawal and infusion were possible and that air emboli were absent. Cannulation of the right jugular vein was carried out using the same procedure.

A 2-cm transverse incision was made through the shaved dorsal surface between the scapulae. A small subcutaneous pocket anterior to the incision was formed by using forceps to loosen the skin from the underlying connection tissue. Using a 16G needle and scalpel, a small opening was made in the skin 1.5 cm anterior to the dorsal incision. A metal coil tether (rodent tether, 18”; Lomir Biomedical Inc., Notre-Dame-de-l’Île Perrot, QC, Canada)
with a plastic button base was tunneled through the incision, into the pocket, and out the
small opening, leaving the button under the skin. The button was sutured to the skin using
3-0 silk sutures (SofSilk, Tyco Healthcare Group Canada Inc., Ville St. Laurent, QC,
Canada), securing the externalized tether. To feed the catheter through the tether, the
catheter was first tunneled subcutaneously on the right side of the neck, from the ventral to
the dorsal incision areas using a 16G needle as a guide. The catheter was fed through the
underside of the plastic button and into the tether such that the end of the catheter
extended out of the tether. The tether and catheter were then secured to the tether-swivel
connector, which was fastened to a clamp on a retort stand. The catheter was tested again
to ensure for proper blood sampling and re-infusion. This rodent tethering system allowed
for manual, undisturbed blood sampling and for unrestricted movement of the rat. The
dorsal incision was closed with discontinuous 3-0 silk sutures. On the ventral side, the
catheter was anchored to the underside of the skin just cranial to the incision line, and the
incision was closed with continuous 3-0 silk sutures.

3.1.4.3 Post-operative care.
Following surgery, incision sites and the dorsal button area were cleaned with 10%
povidone-iodine. Rats were injected subcutaneously with 3.0 mL of saline to prevent
dehydration during recovery from anesthetization. Rats were then allowed to recover in
clean cages lined with paper towel overtop bedding material. The tether extended from the
back of the neck of the rat, through the cage lid, and connected to the swivel apparatus
external to the cage. Rats were allowed at least 3 full days to recover from surgery prior to
experimentation.
3.1.4.4 **Catheter patency and blood sampling.**

To ensure that catheters remained free from clots, the lines were flushed daily with heparinized (10 USP U/mL) saline. Carotid catheters were then filled with 100 USP U/mL heparinized saline, plugged with a saline-filled syringe with a 23-gauge blunted needle, and clamped with hemostats with silastic cuffs. Jugular catheters were filled with 100 USP U/mL heparinized saline, plugged with blunted pins, and secured against the metal tether with masking tape to avoid being caught or pulled.

3.2 **General laboratory methods**

3.2.1 **Blood Glucose Determination**

Whole blood glucose concentrations were measured immediately after obtaining blood samples during experiments by the glucose oxidase method using a glucose analyzer (Analox glucose analyzer, GMD-9D, Analox Instruments USA Inc., Lunenburg, MA). This method was suitable for use with whole blood collected with heparin and/or EDTA. A 10 µL blood sample was pipetted into the reaction well, which contained the glucose oxidase reagent (Glucose oxidase reagent kit, GMRD-020, Analox Instruments USA Inc., Lunenburg, MA). In the presence of oxygen, glucose from the blood sample is oxidized by glucose oxidase to gluconic acid and hydrogen peroxide as follows:

\[ \beta-D\text{-}glucose + O_2 \xrightarrow{\text{glucose oxidase}} D\text{-}gluconic acid + H_2O_2 \]

Oxygen consumption is directly proportional to glucose concentration, and the analyzer measures the rate of oxygen uptake in the reaction well using a Clark-type amperometric oxygen electrode at 30°C. The analyzer is calibrated before the start of, and intermittently
throughout, each experiment using glucose standards of 8.0 (Glucose standard 8.0 mM, GMRD-011) and 2.5 mM (Glucose standard 2.5 mM, RMRD-009), respectively. Results were obtained within 35 sec of sample addition, and blood samples were analyzed in duplicate, ensuring that the measurements obtained were ± 0.2 mM. The analyzer has a linearity of 30 mM and 50 mM using 10 μL and 5 μL samples, respectively.

For blood glucose measurements taken daily to monitor fed morning glycemia, a handheld glucometer (Ascencia Elite, Bayer Canada Ltd., Etobicoke, ON, Canada) was used with blood glucose test strips (Elite, Bayer Bayer Canada Ltd., Etobicoke, ON, Canada). Test strips have been calibrated to give plasma equivalent glucose results. Test strips also use the glucose oxidase method for determinations of glucose concentrations. The range of glucose concentration measurable using test strips was 1.1 to 33.3 mM.

3.2.2 Preparation of blood sample collection tubes
1.5 mL microtubes (Eppendorf; Diamed Lab Supplies Inc., Mississauga, ON, Canada) were used to collect blood samples for various assays. Tubes chilled on ice containing 25 U of dried heparin were used for collection of 0.1 mL of blood for corticosterone assays. For glucagon, catecholamine, insulin, and growth hormone assays, 0.5 mL of blood was collected in chilled tubes containing 50 μL of a 1:1 solution of ethylenediamine tetraacetic acid (EDTA; 0.24 g/mL in distilled water; Sangon Ltd. Canada, Scarborough, ON, Canada) and Trasylol (2000 kallikrein IU; Bayer Canada Ltd., Etobicoke, ON, Canada). For somatostatin assay, two tubes of 1.0 mL blood each were collected in chilled tubes each containing 100 μL of the 1:1 EDTA:Trasylol solution (i.e. 2.0 mL of blood collected in total at euthanasia for somatostatin assay). EDTA is an anticoagulant, and Trasylol is a broad spectrum proteolytic inhibitor that protected the plasma against loss of hormone reactivity. These tubes were kept chilled (4°C) prior to collection of blood.
After blood was collected in the respective tubes, tubes were shaken to ensure adequate mixing of their contents. Blood was then centrifuged (30-60 sec at 9000 rpm) to separate plasma from packed red blood cells. Plasma was then immediately frozen and kept at -80°C.

3.2.3 Radioimmunoassays (RIA) for plasma hormone levels

Plasma corticosterone, glucagon, and catecholamine levels were measured using radioimmunoassay (RIA). This procedure involves a competitive reaction between an unknown amount of antigen (i.e. hormone) present in a plasma sample and a known amount radiolabeled antigen for limited binding sites on an antibody (525). In our RIAs, the radioactive label used was $^{125}$I. After an incubation period between these reagents during which unlabeled and labeled hormones vie for a binding site on the antibody, an equilibrium is reached. This equilibrium is dependent on the relative amounts of unlabeled and labeled hormones and reflects the nature of competitive reactions. The bound hormone-antibody complexes, which are in the form of a precipitant, are then separated from the free hormones, which are in the liquid phase. This separation involves centrifuging the mixtures and removing the liquid phase by decanting or aspirating. The amount of bound radiolabeled hormone is then measured in a gamma scintillation counter. The assay is calibrated by measuring standards of known concentrations and cross-plotting the counts emitted by the radioactive label versus the concentration of the standards to generate a dose-response curve. As the concentration increases, the number of counts decreases exponentially. This is true also for the sample in question; the precipitated complex of a sample carrying a high concentration of unlabeled hormone will have lower radioactivity since there would be less radiolabeled hormone bound to the limited amount of antibody. The reverse is also true in that a sample with a low concentration of unlabeled hormone will
yield a precipitated pellet of lower radioactive signal. Non-specific binding is a control parameter that represents the minimal binding (525). Percent bound values are calculated for both standards and samples in the equation:

\[
\text{Percent bound} = \frac{\left(\text{sample counts} - \text{non-specific binding counts}\right)}{\left(\text{zero calibrator counts} - \text{non-specific binding counts}\right)} \times 100\%
\]

3.2.3.1 *Plasma corticosterone assay.*

ImmunoChem™ Double Antibody Corticosterone \(^{125}\text{I} \) RIA kits (MP Biomedicals, Solon, OH, USA) were used to measure total corticosterone levels in rat plasma. Assay controls were reconstituted with 2 mL of distilled water and allowed to sit at room temperature for at least 30 min before use. Other reagents from the kit and frozen plasma samples were also brought to room temperature. Thawed samples were then centrifuged at \( 4^\circ \text{C} \) to remove fibrin or other particulate matter. Plasma was diluted at a ratio of 1:200 by adding 2 mL of steroid diluent to 10 \( \mu \text{L} \) of plasma. The steroid diluent provided with the kit consisted of phosphosaline gelatin buffer at pH 7.0 ± 0.1 with rabbit gamma globulins.

Six corticosterone standards in concentrations ranging from 0 to 1000 ng/mL were used to produce a standard curve for the assay. 100 \( \mu \text{L} \) of each of the standards, controls, and diluted plasma samples were pipetted into polystyrene tubes. Standards were assayed in triplicate while controls and samples were assayed in duplicate. To each of these tubes, 200 \( \mu \text{L} \) of \(^{125}\text{I} \)-corticosterone followed by 200 \( \mu \text{L} \) of anti-corticosterone antibody was added, vortexed, and incubated at room temperature for 2 h. Corticosterone-3-carboxymethyloxime:BSA was used as the antigen to generate antiserum in rabbits.

Following the incubation, 500 \( \mu \text{L} \) of precipitating solution, a mixture of goat anti-rabbit gamma globulin and polyethylene glycol in TRIS buffer, was added to the tubes. Tubes
were then vortexed and centrifuged at 2500 rpm for 20 min. This allowed for the separation of bound antibody-antigen complexes (pellet) from the free fraction (supernatant). A triplicate of tubes containing only $^{125}$I-corticosterone (to measure total counts) and another triplicate of tubes containing only $^{125}$I-corticosterone and precipitating solution (to measure non-specific binding) also underwent the same incubation and vortexing procedures. The supernatant was then aspirated from all tubes, except for the tube only containing $^{125}$I-corticosterone, and the precipitate was counted in a gamma-scintillation counter to measure their reactivity. A standard curve was produced by plotting percent bound against concentration of corticosterone for the standards. Plasma concentrations of the samples were automatically calculated by computer software linked to the gamma counter. The manufacturer’s assay precision was reported as intra-assay coefficient of variation between 4.4-10.3% and inter-assay coefficient of variation between 6.5-7.1%.

3.2.3.2 Plasma glucagon assay.

Plasma glucagon levels were measured using LINCO/Millipore’s Glucagon RIA Kit (GL-32K, LINCO Research Inc., St. Charles, MO, USA/Millipore Corporation, Billerica, MA, USA). This assay uses $^{125}$I-glucagon and glucagon antibody to determine the level of glucagon in plasma by using the double antibody technique. Samples were thawed just prior to the assay and centrifuged to separate plasma from fibrin or other particulate matter. All reagents, besides the $^{125}$I-glucagon tracer, were provided ready to use. 100 µL of plasma was added with 100 µL of assay buffer, which contained 0.2M glycine, 0.03M EDTA, 0.08% sodium azide, and 1% RIA-grade BSA at a pH 8.8 into polystyrene tubes. When 100 µL of plasma was not available, smaller volumes of sample were used and additional assay buffer was added to compensate for the difference so that
the total sample volume was equivalent to 100 µL, as directed by the kit. Five standards ranging from glucagon concentrations of 20 to 400 pg/mL were included in the kit. A zero standard was produced using 200 µL of assay buffer as instructed by the kit, and a 10 pg/mL standard was made by diluting the 20 pg/mL standard in a 1:1 ratio with assay buffer. 100 µL of each standard and control was pipetted into tubes along with 100 µL of assay buffer. To sample, standard, and control tubes, 100 µL of guinea pig glucagon antibody was added. All tubes were assayed in duplicate. Tubes containing 300 µL of assay buffer and no antibody were included in the assay to measure non-specific binding. Tubes were vortexed, covered, and incubated at 4°C for 20 hours.

Following the incubation period, lyophilized 125I-glucagon tracer was mixed with label hydrating buffer, which had the same composition as assay buffer, and allowed to sit at room temperature for 30 min. All tubes were then pipetted with 100 µL of 125I-glucagon. 100 µL of 125I-glucagon was added to two more tubes to measure total radioactivity (i.e. total count tubes). All tubes were then vortexed, covered, and allowed to incubate at 4°C for an additional 20 hours.

After the second incubation period, 1.0 mL of cold (i.e. 4°C) precipitating solution was added to all tubes except for total count tubes. Tubes were then vortexed and incubated for 20 min at 4°C after which they were centrifuged for 20 min at 4°C. Supernatant was then immediately aspirated from the tubes, except for total count tubes, and pellets containing the antibody-antigen complexes were counted in a gamma-scintillation counter to measure their radioactivity. A standard curve was plotted from the assay standards. Plasma concentrations of the samples were automatically calculated by computer software linked to the gamma counter. Samples which contained less than 100 µL plasma were multiplied by the appropriate factor. The intra-assay coefficient of variation was reported as 4.0-6.8%.
The inter-assay coefficient of variation was reported as 7.3-13.5%. The specificity of the test was reported as 100% for glucagon and <0.1% for oxyntomodulin, which contains the glucagon sequence.

3.2.3.3 Plasma catecholamine assay.

A two-catecholamine RIA kit from Labor Diagnostika Nord (LDN) was used for the quantitative determination of plasma epinephrine and norepinephrine (BA 1500, LDN GmbH & Co. KG; Nordhorn, Germany). In this test, epinephrine and norepinephrine are first extracted by using a cis-diol-specific affinity gel, acylated to N-acylepinephrine and N-acylnorepinephrine, and then enzymatically converted during the detection procedure to N-acylmetanephrine and N-acylnormetanephrine. The subsequent assay procedure follows the basic RIA principle.

All reagents are brought to room temperature before use, except for the precipitating solution which is kept chilled (~ 4°C). Standards, controls, and plasma samples are first extracted. Six standards ranging for each of epinephrine and norepinephrine are provided with the kit in the concentration range of 0 to 90 ng/mL and 0 to 450 ng/mL, respectively. 20 μL of each standard and controls and 80 μL of distilled water are pipetted into wells of the extraction plate, which is coated with boronate affinity gel. 100 μL of sample plasma is also pipetted into separate wells of the extraction plate. 50 μL of each of assay buffer (containing 1 M hydrochloric acid) and extraction buffer are added to all wells, and the plate is covered and incubated at room temperature on an orbital shaker (600-900 rpm) for 30 min. Following the incubation period, standards, controls, and samples are acylated to N-acylepinephrine and N-acylnorepinephrine. The plate is decanted, and 150 μL of acylation buffer followed by 25 μL of acylation reagent are added to all wells. The plate is then incubated under the same conditions as before for 15 min, decanted, pipetted with 1 mL of
distilled water per well, incubated again for 5 min, and decanted again. 200 μL of 0.025 M hydrochloric acid is then added to each well, and the plate is covered and incubated as before for 10 min. This eluate is then ready for RIA.

Epinephrine and norepinephrine RIAs are performed in separate tubes but from the same extracted samples. Thus, the appropriate radioactively labeled antigen and antibody must be used for each assay. 90 μL of the extracted standards, controls, and samples are pipetted into polystyrene tubes for each of the epinephrine and norepinephrine assays. To measure non-specific binding, 90 μL of hydrochloric acid is pipetted into separate tubes. An enzyme provided with the kit containing catechol-O-methyltransferase is reconstituted in 1 mL of distilled water mixed thoroughly. To this solution, 0.3 mL of the coenzyme S-adenosyl-L-methionine is then added, followed by 0.7 mL of the enzyme buffer. This solution must be prepared no longer than 10 min prior to use. 25 μL of enzyme solution is added to all tubes. Tubes are then mixed and incubated at 37°C for 30 min.

After the incubation, 50 μL of ¹²⁵I-epinephrine (or ¹²⁵I-norepinephrine) is added to the tubes. Tubes for measuring total radioactivity (i.e. total counts) are also included in the assay and contain only the radioactive label. 50 μL of epinephrine antibody (or norepinephrine antibody) is then added to all tubes except tubes for non-specific binding or total counts. After tubes are mixed and centrifuged at 500 xg and incubated at 4°C overnight. Cold precipitating reagent is mixed and 1 mL is then added to all tubes (except total counts). Tubes are vortexed and centrifuged for 15 min at 3000 xg at 4°C. Supernatant is then aspirated, leaving behind a pellet containing antibody-antigen complexes. Tubes for each assay were counted separately in a gamma-scintillation counter to measure their radioactivity. A standard curve was plotted from the assay standards. Plasma concentrations of the samples were automatically calculated by computer software.
linked to the gamma counter. The intra-assay coefficient of variation was reported as 4.6-14% and 4.0-4.6% for epinephrine and norepinephrine, respectively. The inter-assay coefficient of variation was reported as 5.6-6.1% and 6.1-10% for epinephrine and norepinephrine, respectively.

3.2.3.4 Plasma somatostatin assay.
The EURIA somatostatin RIA kit was used for the quantitative determination of plasma somatostatin concentrations (RB-306, Euro-Diagnostica AB, Malmo, Sweden). Due to the large volume of plasma needed for this assay, plasma was obtained from trunk blood at euthanasia. Somatostatin from the plasma is first extracted with Sep-Pak C18 cartridges (WAT023635, Waters Ltd., Milford, MA). The extracts are then assayed using $^{125}$-Tyr$^1$-somatostatin and an antibody to synthetic somatostatin-14 to determine the level of somatostatin in the sample using the double antibody technique.

Plasma samples were thawed just prior to the assay and centrifuged to separate plasma from fibrin or other particulate matter, and 0.5 mL of plasma was aliquoted into eppendorf tubes. 50 μL of 1M hydrochloric acid was added to each sample and vortexed. The Sep-Pak cartridge was wetted with 5 mL of methanol and washed with 20 mL of distilled water. Separate cartridges were used for each sample. At a flow rate no greater than 1 mL/10 sec, the plasma-acid solution was applied to the Sep-Pak cartridge and then washed with 20 mL of 4% acetic acid in distilled water. Somatostatin was subsequently eluted from the cartridge with 2 mL of methanol also at a flow rate less than 1 mL/10 sec and collected in a 10 mL glass tube. The eluate was then dried in an evaporator. Extracted somatostatin dissolved in 0.5 mL of assay diluents, which contained 0.05 M phosphate buffer at pH 7.4, 0.25% human serum albumin, 0.25% EDTA, 0.05% sodium azide, 0.1% Tween 80, and 500
KIU/mL Trasylol. Reconstituted samples were vortexed thoroughly and rested for 30 minutes prior to the RIA procedure.

For determination of the recovery in the extraction procedure, two recovery controls were prepared. Control A consisted of 0.8 mL of rat plasma combined with 80 µL of 1M hydrochloric acid, to which 200 µL of the 250 pmol/L somatostatin standard was added to yield a final concentration of 50 pmol/L. Control B consisted of 0.8 mL of the same rat plasma combined with 80 µL of 1M hydrochloric acid, to which 200 µL of assay diluents was added. Both controls were extracted using the same procedure described above for the samples. Control B was used for the correction for endogenous somatostatin in the calculation of the recovery of added somatostatin.

A 250 pmol/L synthetic cyclic somatostatin (MW = 1638.1) standard was prepared in a solution with same composition as the assay diluent and lyophilized. This lyophilized standard was reconstituted in 5 mL distilled water and serially diluted with assay diluent to yield 7 standard solutions ranging from 0 to 125 pmol/L. Rabbit somatostatin antibody was raised against cyclic somatostatin conjugated to bovine thyroglobulin. The antibody and ¹²⁵⁻somatostatin were prepared in a solution with same composition as the assay diluent and lyophilized, and both were reconstituted in 22 mL and 25 mL of distilled water, respectively, prior to the RIA procedure. 100 µL of standards, controls, and reconstituted samples were pipetted into polystyrene tubes in duplicate. An additional pair of tubes with 300 µL of assay diluent was included to measure non-specific binding. 200 µL of somatostatin antibody was added to tubes containing standards, controls, and reconstituted samples and vortexed. After a 20-h incubation at 4°C, 200 µL of ¹²⁵⁻somatostatin was added to all tubes and vortexed. Two additional tubes of 200 µL of ¹²⁵⁻somatostatin were included to measure total count of radioactivity. After a second 20-h incubation at 4°C, 100 µL of precipitating
reagent was added to all tubes except for total counts. This reagent consisted of rabbit immunoglobulins coupled to cellulose particles and since it was a suspension, it required continuous stirring during pipetting. Tubes were vortexed thoroughly, incubated for 45 minutes at 4°C, and centrifuged at 2500 rpm at 4°C for 20 min. Supernatant was then immediately aspirated from the tubes, except for total count tubes, and pellets containing the antibody-antigen complexes were counted in a gamma-scintillation counter to measure their radioactivity. A standard curve was plotted from the assay standards. Plasma concentrations of the samples were automatically calculated by computer software linked to the gamma counter.

Somatostatin recovery was calculated as follows:

\[
\text{% recovered somatostatin} = \left[ \frac{(\text{concentration Control A}) - (\text{concentration Control B})}{50} \right] \times 100\%
\]

The recovery should be at least 60% for a valid assay. Sample concentrations were then corrected for the percent recovery and also multiplied by a factor of 1.1 to correct for the increased sample volume by adding 1M hydrochloric acid. The intra-assay coefficient of variation was reported as 2.8-8.3%. The inter-assay coefficient of variation was reported as 3.3-6.4%.

3.2.3.5 Plasma insulin assay

Plasma insulin levels were measured using LINCO/Millipore’s Rat Insulin RIA Kit (RI-13K, LINCO Research Inc., St. Charles, MO, USA/Millipore Corporation, Billerica, MA, USA). This assay uses \(^{125}\text{-insulin}\) and a rat insulin antibody to determine the level of rat insulin in plasma by using the double antibody technique.
Samples were thawed just prior to the assay and centrifuged to separate plasma from fibrin or other particulate matter. Since high doses of insulin were administered in the hypoglycemia experiments, plasma samples obtained after insulin administration had to be diluted with assay buffer before assay. For Study 1, when 10 U of insulin was injected, plasma samples at time 60 and 180 min were diluted 100 and 20 times, respectively. For Study 2, plasma samples at time 60 were diluted 100 times.

All reagents, besides the $^{125}$I-rat insulin tracer, were provided ready to use. One-half of the volumes of all reagents suggested in the manufacturer’s protocol were used in this assay. 50 µL of plasma (or diluted plasma) was pipetted into glass sample tubes. 100 µL and 50 µL of assay buffer, which contained 0.05 M phosphosaline at pH 7.4, 0.025M EDTA, 0.08% sodium azide, and 1% RIA-grade BSA, was added to the non-specific binding tubes and reference tubes, respectively. Seven standards ranging from rat insulin concentrations of 0.1 to 10.0 ng/mL were included in the kit. 50 µL of each standard and control was pipetted into tubes. All tubes were assayed in duplicate. Lyophilized $^{125}$I-insulin tracer was hydrated with 27 mL of hydrating buffer and rested at room temperature for 30 min with occasional gentle mixing. The hydration buffer contained normal guinea pig immunoglobulin G. 50 µL of hydrated $^{125}$I-insulin was added to all tubes. 50 µL of $^{125}$I-insulin was added to two more tubes to measure total radioactivity (i.e. total count tubes). To sample, standard, and control tubes, 50 µL of guinea pig rat insulin antibody was added. The rat insulin antibody was raised in guinea pigs against highly purified rat insulin. All tubes were assayed in duplicate. Tubes were vortexed, covered, and incubated at 4°C for 20 hours.

After the incubation period, 0.5 mL of cold (i.e. 4°C) precipitating solution was added to all tubes except for total count tubes. The precipitating solution contained goat anti-guinea pig immunoglobulin G serum, 3% polyethylene glycol, 0.05% TritonX-100 in 0.05 M
phosphosaline, 0.025 M EDTA, and 0.08% sodium azide. Tubes were then vortexed and incubated for 20 min at 4°C after which they were centrifuged for 20 min at 4°C. Supernatant was then immediately aspirated from the tubes, except for total count tubes, and pellets containing the antibody-antigen complexes were counted in a gamma-scintillation counter to measure their radioactivity. A standard curve was plotted from the assay standards. Plasma concentrations of the samples were automatically calculated by computer software linked to the gamma counter. Samples which were diluted were multiplied by the appropriate factor. The intra-assay coefficient of variation was reported as 1.4-1.6%. The inter-assay coefficient of variation was reported as 8.5-9.4%. The specificity of the test was reported as 100% for rat, human, and porcine insulin and not detectable for IGF-I or rat C-peptide.

3.2.3.6 Plasma growth hormone assay
Plasma growth hormone levels were measured using LINCO’s Rat Growth Hormone RIA Kit (RGH-45HK, LINCO Research Inc., St. Charles, MO, USA). This assay uses 125I-rat growth hormone and a rat growth hormone antibody to determine the level of rat growth hormone in plasma by using the double antibody technique.

Samples were thawed just prior to the assay and centrifuged to separate plasma from fibrin or other particulate matter. All reagents, besides the 125I-growth hormone tracer, were provided ready to use. 100 μL of plasma was pipetted into sample tubes containing 100 μL assay buffer. The assay buffer contained 0.05 M phosphosaline at pH 7.4, 0.025M EDTA, 0.08% sodium azide, and 1% RIA-grade BSA. 300 μL and 200 μL of assay buffer was added to the non-specific binding tubes and reference tubes, respectively. Seven standards ranging from rat growth hormone concentrations of 0.5 to 50.0 ng/mL were included in the kit. 100 μL of each standard and control was pipetted into tubes. All tubes were assayed in
duplicate. Lyophilized $^{125}$I-growth hormone tracer was hydrated with 13.5 mL of hydrating buffer and rested at room temperature for 30 min with occasional gentle mixing. The hydration buffer contained normal guinea pig serum. 100 µL of hydrated $^{125}$I-growth hormone was added to all tubes. 100 µL of $^{125}$I-growth hormone was added to two more tubes to measure total radioactivity (i.e. total count tubes). Both the $^{125}$I tracer and standards were prepared with recombinant rat growth hormone. To sample, standard, and control tubes, 100 µL of guinea pig rat growth hormone antibody was added. The rat growth hormone antibody was raised in guinea pigs against recombinant rat growth hormone. All tubes were assayed in duplicate. Tubes were vortexed, covered, and incubated at 4°C for 20 hours.

After the incubation period, 1.0 mL of cold (i.e. 4°C) precipitating solution was added to all tubes except for total count tubes. The precipitating solution contained goat anti-guinea pig immunoglobulin G serum, 3% polyethylene glycol, 0.05% TritonX-100 in 0.05 M phosphosaline, 0.025 M EDTA, and 0.08% sodium azide. Tubes were then vortexed and incubated for 20 min at 4°C after which they were centrifuged for 20 min at 4°C. Supernatant was then immediately aspirated from the tubes, except for total count tubes, and pellets containing the antibody-antigen complexes were counted in a gamma-scintillation counter to measure their radioactivity. A standard curve was plotted from the assay standards. Plasma concentrations of the samples were automatically calculated by computer software linked to the gamma counter. The specificity of the test was reported as 100% for rat growth hormone and <0.1% for rat prolactin and human and porcine growth hormone.
3.2.4 Pancreatic protein determination

Pancreatic glucagon and somatostatin protein content was measured from pancreata collected from animals in Study 1. Pancreata were quickly removed at euthanasia, frozen in sterile tubes on dry ice, and stored at -80°C.

3.2.4.1 Pancreas homogenization for protein isolation

Frozen pancreata were individually homogenized in 3 10-second intervals on medium-high speed in 50-mL falcon tubes kept on ice in 10 mL of extraction medium containing 9.2% hydrochloric acid, 5% formic acid, 1% trifluoroacetic acid (TFA), and 1% sodium chloride. Homogenates were centrifuged at 2300 rpm at 4°C for 15 minutes. The supernatant was collected in a separate falcon tube. An additional 10 mL of extraction medium was added to the remaining pellet, re-homogenized as before, and centrifuged again at 2300 rpm at 4°C for 15 minutes. The second supernatant was collected in the same tube as the first (total final volume of 20 mL), and the pellet was discarded.

3.2.4.2 Sep-Pak protein extraction

Pancreatic protein was isolated by eluting the supernatant of pancreatic homogenates using Sep-Pak C18 cartridges (WAT023635 Waters Ltd., Milford, MA). Separate cartridges were used for each sample. Using a 20 mL syringe, each Sep-Pak cartridge was first wetted with 15 mL of a solution containing 80% isopropanol and 0.1% TFA, followed by 15 mL of 0.1% TFA. The 20 mL of supernatant from the homogenized pancreata were then slowly passed through the Sep-Pak twice. Each Sep-Pak cartridge is subsequently washed using 15 mL of 0.1% TFA to remove any unbound sample. At the end of this stage only is air allowed to pass through the column to remove all liquid (approximately 2 mL of air from a syringe). The protein is then eluted from the column using 10 mL of solution containing 80% isopropanol and 0.1% TFA into a 15 mL falcon tube. Pancreatic protein eluates were frozen
at -80°C until used. For glucagon and somatostatin determinations using commercially available radioimmunoassay kits, 1-mL aliquots of these eluates were evaporated, and isolated protein was re-dissolved in 3 mL of double-distilled water. Glucagon and somatostatin measurements were normalized for total protein content as determined by spectrophotometer analysis.

3.2.5 Liver mRNA expression quantification

Livers were quickly removed at euthanasia, frozen in sterile tubes on dry ice, and stored at -80°C.

3.2.5.1 Total RNA preparation.

Total RNA was extracted from frozen liver using a modified phenol-chloroform method with TRI Reagent® (TR-118, Sigma-Aldrich, St. Louis, MO, USA). TRI Reagent® combines phenol and guanidine thiocyanate to facilitate inhibition of RNAse activity. A small sample of liver (approximately 2 x 2 x 2 mm³) was homogenized in a 15 mL snap cap tube for 20-30 on medium-high speed in a fume hood. The homogenate was then incubated at room temperature for at least 5 min. 800 µL of chloroform was then added to the homogenate and shaken vigorously before incubating for an additional 5 min at room temperature. Using a 1 mL pipette, the upper phase (approximately 2 mL of clear fluid) of the mixture was carefully collected in a new 15 mL snap cap tube with care being taken to avoid the buffy white layer to avoid DNA contamination. At this stage, RNA remains exclusively in the aqueous phase (supernatant), DNA in the interphase (buffy white layer), and proteins in the organic phase (pellet). 2 mL of isopropanol was then added to the supernatant collected, and the tube was mixed by inversion and incubated at room temperature for 15 min. Tubes containing the samples were centrifuged at 12 000 g at 4°C for 10 min. The orientation of the tubes was noted prior to centrifugation so that the small RNA pellet could be easily
identified following centrifugation. The supernatant was then removed by aspiration, and 4 mL of ice-cold 75% ethanol was added to the remaining pellet. Tubes were then vortexed thoroughly and centrifuged at 10 000 g at 4°C for 5 min. The supernatant was then carefully removed by aspiration. Tubes were then allowed to vent at room temperature for 30 min to evaporate off any remaining ethanol. Pellets were resuspended in 100 μL diethylpyrocarbonate (DEPC) water (i.e. RNAse free water) by repeated drawing and expelling the pellet with a pipette tip.

Quantification of RNA content was performed by measuring the optical density (OD) at 260 and 280 nm using 2 μL of sample with a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Mississauga, ON, Canada). For RNA, the ratio of 260/280 should be between 1.8 and 2. OD measurements below this ratio are indicative of contamination with protein or solvents. RNA concentration (in μg/mL) was then calculated as:

\[
\text{RNA concentration} = \text{OD}_{260} \times \text{dilution factor} \times 40
\]

The resuspended RNA sample was transferred to eppendorf tubes and stored at -80°C until use.

3.2.5.2 cDNA synthesis.

Frozen RNA dissolved in DEPC water was heated in a water bath at 65°C for 5 min. Using concentrations obtained from spectrophotometer determinations, 10 μg of RNA was very gently combined with 5 μL DEPC water, 2 μL DNAse buffer (10x), and 3 μL DNAse in sterile microtubes. After a 5-min incubation at room temperature, the DNAse reaction was stopped with incubation with 2 μL 25mM EDTA at 70°C for 12 min.
Following treatment with DNase I, purified RNA was reverse transcribed to synthesize first-strand cDNA using SuperScript™III (Invitrogen Corporation, Carlsbad, CA, USA). 2 µL of random primers (12-times dilution in DEPC water; 48190-011, Invitrogen Corporation) 2 µL 10 mM dNTP (Fermentas, Thermo Fisher Scientific, Mississauga, ON, Canada) were added to the DNAse-treated RNA and heated at 65°C for 5 min and cooled on ice for 1 min. 8 µL First-Strand buffer (5x, Invitrogen Corporation) and 2 µL 0.1 M DTT (Invitrogen Corporation) were added to the tubes. The contents of each tube (total volume = 36 µL) was then equally divided into 2 sets of tubes (18 µL per tube): RT+ tubes to which 1 µL of SuperScript™III Reverse Transcriptase (Invitrogen Corporation) was added and RT- tubes (a negative control for reverse transcription), to which 1 µL DEPC water was added. The tubes were then incubated at 25°C for 10 min (reaction for random primers), 50°C for 60 min (reverse transcription reaction), and 70°C for 15 min (inactivation of reaction) in a thermal cycler (Vapo Protect Mastercycler® pro, Eppendorf Canada, Mississauga, ON, Canada). RNA complementary to the cDNA was then removed by adding 1 µL RNAse H (2.5-times dilution in RNAse buffer; Fermentas) and incubating at 37°C for 20 min. The cDNA can then be used as a template for amplification.

3.2.5.3 Polymerase chain reaction (PCR) of β-actin.

This PCR was used to determine the integrity of the RT+ and RT- sets of synthesized cDNA. Samples from RT+ tubes should demonstrate amplification of the DNA of interest (β-actin), whereas RT- tubes which lacked reverse transcriptase enzyme should not amplify DNA. Signals that appear in the RT- tubes are indicative of DNA contamination. A PCR mixture is first made for all samples to be run by combining the following reagents using the following ratio per 1 µL cDNA sample: 11 µL double-distilled water, 0.25 µL 5’ (forward) primer for β-actin (ACGT Corp, Toronto, ON, Canada), 0.25 µL 3’ (reverse) primer for β-actin (ACGT
Corp), and 12.5 μL REDTaq® (Sigma-Aldrich, St. Louis, MO, USA). Care should be taken to gently combine the REDTaq® solution, which contains the enzyme Taq DNA polymerase. In 96-welled plates, 1 μL of cDNA from each of RT+ and RT- sets of tubes was first pipetted before 24 μL of the PCR mixture was then added to each well. Plates were then spun at 1000 rpm at 4°C for 30 sec. After thermal cycler PCR machine was warmed up, plates were sealed with a film to prevent evaporation and placed in the thermal cycler at 94°C for 5 min (initial denaturation) and cycled 40 times at 94°C for 30 sec, 45°C for 30 sec, and 72°C for 30 sec (amplification) prior to incubation at 72°C for 4 min (final elongation), and then 4°C for storage.

A 1% agarose running gel for β-actin was prepared by combining 200 mL 1x TBE (Tris, boric acid, and EDTA), 2 g agarose powder, and 20 μL SYBR® Safe (10 000x, Invitrogen Corporation) DNA gel stain, heating until completely dissolved, and setting into a 20-comb cast. The first well of each row was reserved for 10 μL of DNA ladder (GeneRuler™, 1 kb, Fermentas), and 10 μL of sample was loaded into the wells. Charge was applied to the gel at approximately 90 V, and samples travelled through the gel from the negative to positive electrode. Blots on finished gels were visualized under ultra violet light in a UVP BioDoc-It System (UVP, Upland, CA, USA). Real-time quantitative PCR was run only for samples that showed no contamination.

### 3.2.5.4 Real-time quantitative PCR.

Real-time quantitative PCR was run only for samples that showed no contamination. Several RT- samples and a DEPC-water sample were included in the run to serve as controls. All samples were run in duplicate. 1 μL of cDNA and 4 μL of yellow-coloured DEPC water was pipette into wells of MicroAmp® Optical 384-Well Reaction Plates (Applied Biosystems, Foster City, CA, USA). The yellow colouring allowed for easier detection when
working with such small volumes. For cyclophilin, cDNA was first diluted 50 times prior to adding 1 μL of the diluted cDNA to the wells. Real-time quantitative PCR was performed for using primers for 2 genes of interest (PEPCK1 (Pck1), Mm01247058_m1; G6Pase (G6pc), Mm00839363_m1) and 1 housekeeping gene (cyclophilin (Ppia), Mm02342430_g1) using TaqMan Gene Expression Assays (Applied Biosystems). A reaction mixture of TaqMan® Universal PCR Master Mix (Applied Biosystems), primer of the gene, and blue-coloured DEPC water was combined together in a ratio of 10:1:4 prior to pipetting 15 μL of this mixture into each well. Plates were sealed with MicroAmp® Optical Adehesive Film (Applied Biosystems) and spun at 1000 for 1 min at 4°C.

Real-time quantitative PCR was performed with ABI Prism 7900 Sequence Detection System with Sequence Detection System v2.1 software (Applied Biosystems). The thermal cycling conditions for the reaction consisted of 2 min at 50°C, 10 min at 95°C, and 40 cycles of 15 sec at 95°C and 1 min at 60°C. After plates were run, threshold cycle (Ct) values were analyzed. Ct values should be between 20-25; cDNA needs to be diluted if Ct values are below this range. If Ct values fall above this range, cDNA needs to be more concentrated, or optimization of the primer is necessary. Ct values for the samples run were within the optimal range. The expression values of each gene of interest were normalized to the internal control gene, cyclophilin, for the efficiency of amplification and quantified by the 2^{-ΔΔCt} method (526). Cyclophilin expression values did not vary between experimental groups, deeming it a suitable control gene.
3.3 Preparation of SSTR2 antagonist and saline infusates

This peptide (referred to in literature by the names PRL-2903, DC-41-33, and BIM-23458) was synthesized and provided by Dr. David Coy (Tulane University, New Orleans, LA, USA) in lyophilized form. The peptide was stored at -20°C in a jar of desiccant. To prepare stock-solutions for long-term storage, the peptide was carefully weighed and aliquoted into eppendorf tubes, and dissolved in a calculated volume of 1% acetic acid to a concentration of approximately 25 000 nmol/mL. Each vial of aliquot was carefully labeled for its concentration, sealed with parafilm, and stored also at -20°C. Repeated thawing and refreezing was avoided. On the morning of the experiment, a vial of aliquoted peptide stock solution was thawed on ice and diluted with 0.9% saline (NaCl solution) specific to the dose used for each experiment (1500 nmol/kg/h in Study 1 or 3000 nmol/kg/h in Study 1 and Study 2). In Study 1, 5 mL of infusate was prepared per animal, and in Study 2, 6 mL of infusate was prepared per animal. This volume was used to allow for enough infusate for the 4 or 5 h infusion period for each of Study 1 and Study 2, respectively, at an infusion rate of 1 mL/h in addition to the volume needed to prime the infusion lines. Infusates were neutral in pH. Rats that did not receive SSTR2 antagonist were given infusions of vehicle solution (i.e. 1% acetic acid in 0.9% saline at a ratio of approximately 250 μL : 5 mL).

3.4 Statistical analysis

Statistical analysis was performed using Statistica software (Statsoft Inc., Tulsa, OK, USA). In all tests, significance was deemed when \( P < 0.05 \). Unless otherwise stated, all data are expressed as mean ± standard error of the mean (SEM). Outlying data points were removed when they occurred greater than two standard deviations from the mean.
One-Way Analysis of Variance (ANOVA) was used to compare metabolic parameters, basal hormone values, hormone responses, areas under the curve (AUC), and tissue mRNA and protein levels between groups, unless otherwise stated. AUC was calculated using Prism software (GraphPad Software, San Diego, CA, USA). Measurements that were taken repeatedly over time were compared using Repeated Measures ANOVA. If differences between groups were significant using ANOVA, Duncan’s post-hoc test was used to determine which groups differed from each other. In Study 2, comparisons within the same group on Experimental Day 1 and Experimental Day 2 were assessed using a paired $T$-test.
4 Study 1

4.1 Aims

The objective of this study was to determine whether the glucagon response to hypoglycemia can be improved or normalized in diabetic rats by removing the suppressive effect of somatostatin on glucagon secretion. Different subtypes of the somatostatin receptor within the pancreatic islet mediate the inhibitory effects of somatostatin on islet hormone secretion. In rodent and human islets, somatostatin receptor type 2 (SSTR2) are found nearly exclusively on glucagon-secreting alpha-cells while SSTR1 and SSTR5 are abundant on human and human and rodent beta-cells, respectively (385;389). In this study, we use an antagonist to SSTR2, via which somatostatin exerts its inhibitory effect on stimulated glucagon secretion. Using this selective antagonist to SSTR2, Efendic and colleagues demonstrated that stimulated-secretion of glucagon is dose-dependently enhanced in perifused islets and perfused pancreata of non-diabetic rats. Increasing somatostatin concentrations dose-dependently reverses the effectiveness of the SSTR2 antagonist, which demonstrates a competitive nature for the SSTR2 (392). We hypothesize that in diabetes during hypoglycemia, glucagon release is, at least in part, suppressed by the prevailing inhibitory effect of excessive somatostatin acting via SSTR2. Thus, we hypothesize that selective SSTR2 antagonism will normalize the glucagon response to hypoglycemia.
Somatostatin also inhibits the release of other counterregulatory hormones: the hypothalamic-pituitary-adrenal (HPA) axis (corticotrophin-releasing hormone (CRH) and adrenocorticotropic hormone (ACTH)) and the sympathoadrenal system (epinephrine and norepinephrine) (271). Intracerebroventricular administration of somatostatin or its analogues decreases stress-induced glucocorticoid and catecholamine responsiveness (169;527;528). Thus, we also evaluated the effect of SSTR2 antagonism on other counterregulatory hormone responses during hypoglycemia. Presently, we demonstrate that SSTR2 antagonism restores glucagon and corticosterone counterregulation to hypoglycemia in diabetic rats. In the absence of hypoglycemia, this SSTR2 antagonist neither elicits hyperglycemia nor markedly elevates counterregulatory hormone levels. Finally, we wished to determine whether improved glucagon and corticosterone counterregulation could: 1) reduce the potential risk of hypoglycemia in an unclamped condition as would be indicated by a decrease in the exogenous glucose requirement during our conditions of hypoglycemic clamp; and 2) increase the mRNA expression of gluconeogenic enzymes, phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase), despite the large dose of insulin necessary to induce hypoglycemia in our diabetic rats.

4.2 Experimental methods

4.2.1 Study design

The experimental protocol of Study 1 is shown in Figure 4-1. Three main groups of rats – non-diabetic controls (N), diabetic controls (D), and diabetic rats given somatostatin receptor type 2 antagonist (D+SSTR2a) – were used in hypoglycemia clamp experiments and in control experiments during basal conditions (i.e. in the absence of exogenously
administered insulin). An additional group to assess the effects of SSTR2a during hypoglycemia in non-diabetic rats (N+SSTR2a) was also included. Schematics to illustrate the procedures for hypoglycemia clamp and control experiments are shown in Figure 4-2A and Figure 4-2B, respectively.

4.2.2 Hypoglycemia experiments

After basal samples were obtained at t=-60 min, infusions of saline or SSTR2 antagonist (3000 nmol/kg/h) at 1 mL/h were started for controls and D+SSTR2a rats, respectively. Infusions were delivered via digital pumps (Harvard Apparatus PHD 22/2000 syringe pumps, Holliston, MA). A SSTR2 antagonist dose of 1500 nmol/kg/h was initially tested in our pilot studies based on unrelated in vivo studies in non-diabetic rats using the same antagonist (508). However, since the effect of the SSTR2 antagonist at 1500 nmol/kg/h to enhance glucagon release during hypoglycemia was only moderate in diabetic rats, we rationalized that a larger dose of the antagonist may be necessitated since diabetic rats have increased somatostatin. Blood glucose was measured a glucose analyzer in duplicate at the time points -60, -40, -20, 0, 5, 10, 15, 20, and every 10 min thereafter until 180 min. Blood samples were obtained from the carotid catheter at regular time intervals throughout the glucose clamp and from trunk blood at euthanasia for measurement of plasma hormone levels. 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, regular insulin (10 U/kg; Humulin R, Eli Lilly, Indianapolis, IN) was injected subcutaneously in all rats (N, n=14; D, n=14, D+SSTR2a, n=18). In this study, we chose to give a subcutaneous injection of insulin rather than insulin infusion to induce hypoglycemia. This method of insulin administration is more similar to the clinical insulin regime where hypoglycemia is more relevant. Also, we have
noticed a more marked impairment of the glucagon response to hypoglycemia using insulin injection (174) than insulin infusion (529), despite a similar amount of insulin administered, and we wished to test the efficacy of our SSTR2 antagonist in a setting of greater glucagon unresponsiveness. However, this method of insulin bolus rather than infusion does not result in a steady state of insulin action, and therefore it makes it difficult to measure glucose turnover precisely, which is why we did not use glucose tracer techniques. To evaluate the effect of a lower insulin dose with the same SSTR2 antagonist dose (3000 nmol/kg/h) on glucose infusion rates, hypoglycemia was also induced using 5 U/kg (N, n=5; D, n=4, D+SSTR2a, n=4). Glucose infusions (50% dextrose) were given at a variable rate to clamp glycemia at a target hypoglycemia of 2.5±0.5 mM. After t=180 min, rats were quickly euthanized by decapitation. Plasma from trunk blood and organs were collected, frozen immediately, and stored at -80°C until assayed.

4.2.3 Control experiments: SSTR2 antagonist in the absence of hypoglycemia
To evaluate the effect of SSTR2 antagonist infusion per se, glycemia and plasma glucagon, corticosterone, and catecholamines were measured in the absence of hypoglycemia in a separate set of non-diabetic (Ctrl:N, n=7), diabetic (Ctrl:D, n=7), and diabetic rats given somatostatin receptor type 2 antagonist (Ctrl:D+SSTR2a, n=7) under basal conditions for 240 min. The same methodology and sampling procedure was used during these control experiments with the following exceptions: saline rather than insulin was injected at t=0, and glucose infusions were not used. SSTR2 antagonist was infused at 3000 nmol/kg/h in these control experiments (Ctrl:D+SSTR2a).

4.2.4 Effect of SSTR2 antagonist in non-diabetic rats during hypoglycemia
The effect of SSTR2 antagonism in non-diabetic rats was also assessed during hypoglycemia. This control group allows (N+SSTR2a) for an assessment of the diabetic
impairment that might be attributable to elevated somatostatin. Using the same protocol as the hypoglycemia experiments described above, glucagon and corticosterone responses to hypoglycemia in non-diabetic rats under conditions of high insulin dose (10 U/kg) and 1500 (n=3) and 3000 (n=4) nmol/kg/h SSTR2 antagonist and at low insulin dose (5 U/kg) and 3000 nmol/kg/h (n=10) were assessed and compared to the corresponding non-diabetic controls (N) described above.

4.3 Results

4.3.1 Daily blood glucose, body weight, and food intake

Metabolic parameters such as fed morning blood glucose, body weight, and food intake were measured daily to monitor the health of all rats and to ensure that diabetic rats were of a similar diabetic state leading up to the experimental day before being randomly assigned into control or SSTR2 antagonist treatment groups. Fed morning glycemia significantly increased 4-fold in D compared with N in all three hypoglycemia (including the pilot study) \((P<0.0002)\) and control \((P<0.0002)\) studies (Table 4-1). Diabetic groups within each study had similar hyperglycemia leading up to the clamps. Similarly, D had significantly decreased body weight as compared to N in all three hypoglycemia \((P<0.03)\) and control \((P<0.03)\) studies and similar body weight to D+SSTR2a (Table 4-1). Average daily food intake also significantly increased in D compared with N in all hypoglycemia \((P<0.0002)\) and control \((P<0.0001)\) studies (Table 4-1). D+SSTR2a rats in the hypoglycemia study group with the dosing combination of 10 U/kg insulin and 3000 nmol/kg/h had a slightly lower food intake \((P<0.04)\) as compared to D, despite having similar basal glycemia and body weight. The metabolic changes associated with 3-weeks of diabetes (i.e. severe postprandial hyperglycemia and decreased body weight compared to
non-diabetic controls despite hyperphagia) have been well-documented and represent a
slew of physiologic processes which, taken together, can be summarized as accelerated
catabolism. Simply stated, the metabolic derangements observed in diabetic subjects result
in large from a state of relative insulin deficiency, which leads to catabolic processes
including gluconeogenesis, lipolysis, ketogenesis, and proteolysis (530).

4.3.2 Hypoglycemia clamp experiments

4.3.2.1 Pilot study: using 10 U/kg insulin and 1500 nmol/kg/h SSTR2 antagonist.

In our pilot studies, we initially used SSTR2 antagonist at a dose of 1500 nmol/kg/h, a dose
based on previous in vivo studies in non-diabetic rats (508). SSTR2 antagonist at a dose of
1500 nmol/kg/h in our initial pilot studies yielded significant but very modest improvements
of the glucagon response (Figure 4-3A) and modest and insignificant increases of
corticosterone (Figure 4-3B). We speculated that a larger dose of SSTR2 antagonist would
be required to overcome the increased amounts of pancreatic and circulating somatostatin
observed in STZ-diabetic rats and doubled the dose of SSTR2 antagonist in subsequent
experiments.

4.3.2.2 Using 10 U/kg insulin and 3000 nmol/kg/h SSTR2 antagonist.

D had elevated basal plasma glucagon ($P<0.004$), corticosterone ($P<0.006$), and
epinephrine ($P<0.04$) levels as compared with N, and both groups of diabetic rats had
similar basal plasma hormone levels before administration of SSTR2 antagonist (Table 4-2).
Basal plasma insulin in D was reduced to one-half of the levels in N ($P<0.05$) and similar in
both diabetic groups (Table 4-3).

In the hypoglycemia studies, all groups reached target hypoglycemia ($2.5 \pm 0.5$ mM) by 70
min after insulin injection and remained there until the end of experiment at 180 min
(Figure 4-4). N rats reached target hypoglycemia at time 50 min slightly before the diabetic groups.

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). The glucagon response in D+SSTR2a was fully normalized ($P<0.0001$ vs D; Figure 4-5A). Similarly, the corticosterone response was significantly blunted in D as compared to the 10-fold increase observed in N ($P<0.002$) but was greatly improved in D+SSTR2a ($P<0.05$ vs D; Figure 4-5B).

In the high-dose insulin hypoglycemia clamps, glucose infusion rates (mean infusion rates N: 8.1±1.0; D: 8.9±1.2; D+SSTR2a: 9.2±1.2 mg/kg/min) nor the total amount of glucose infused to maintain target hypoglycemia (Figure 4-5C) differed amongst groups.

N rats exhibited a 69-fold increase in plasma epinephrine, and interestingly, D rats attained a similar plasma epinephrine level to N rats, suggesting that in this model of hypoglycemia, diabetic rats do not exhibit a defect of epinephrine counterregulation (Figure 4-6A). SSTR2 antagonist administration did not alter the epinephrine response to hypoglycemia (Figure 4-6A), presumably because the maximal epinephrine response was already achieved. Norepinephrine levels were likewise similar in all groups, achieving 3- to 5-fold increases in responses to hypoglycemia (Figure 4-6B).

Plasma insulin levels after high-dose (10 U/kg) insulin injection increased approximately 144- to 200-fold in all three groups due to the large amount of insulin required to induce hypoglycemia in diabetic rats which are otherwise quite insulin resistant (Table 4-3). The same dose was used in non-diabetic rats to allow for more relevant comparisons with diabetic groups.
4.3.2.3  **Using 5 U/kg insulin and 3000 nmol/kg/h SSTR2 antagonist.**

An overnight fast lowered initial starting blood glucose levels in both diabetic groups to similar euglycemic levels as non-diabetic rats, despite marked fed glycemia (Table 4-1) and basal plasma insulin at half the concentration of non-diabetic rats (Table 4-3). In the low dose insulin hypoglycemia studies, all groups reached target hypoglycemia (2.5 ± 0.5 mM) 30 min after insulin injection and remained there until the end of experiment at 180 min (Figure 4-7).

Basal plasma glucagon levels were increased in both diabetic groups as compared with non-diabetic rats (Table 4-3). However, basal plasma corticosterone, epinephrine, and norepinephrine were similar in all groups (Table 4-3). D rats also demonstrated markedly attenuated glucagon (*P*<0.04; Figure 4-8A) and corticosterone (*P*<0.04; Figure 4-8B) responses to hypoglycemia induced with a lower dose (5 U/kg) of insulin. D+SSTR2 exhibited fully restored glucagon (*P*<0.005; Figure 4-8A) and corticosterone (*P*<0.04; Figure 4-8B) responses. Thus, the SSTR2 antagonist was effective in improving glucagon and corticosterone counterregulation to hypoglycemia induced by both the high and low doses of insulin tested.

In the low-dose insulin hypoglycemia clamps, D+SSTR2a required lower glucose infusion rates (mean infusion rates: N: 14.1±1.4; D: 15.5±1.5; D+SSTR2a: 6.2±1.2 mg/kg/min; *P*<0.001, D vs D+SSTR2a) and had a lower total glucose requirement than D (*P*<0.008; Figure 4-8C).

N exhibited an 87-fold increase in plasma epinephrine, and interestingly, D attained epinephrine levels similar to N, suggesting that 3-week STZ-diabetic rats do not exhibit a defect of epinephrine counterregulation (Figure 4-9A). Norepinephrine levels were likewise
similar in all groups, achieving 2- to 3-fold increases in response to hypoglycemia (Figure 4-9B).

Plasma insulin levels after low-dose (5 U/kg) insulin injection increased to approximately 60 ng/mL in N, D, and D+SSTR2a (Table 4-3).

Basal plasma growth hormone levels were significantly lower in D as compared to N (P<0.01), and both diabetic groups had similar basal plasma growth hormone levels (Figure 4-10). Plasma growth hormone levels were similar in all groups during hypoglycemia. Notably, administration of SSTR2 antagonist in diabetic rats did not affect growth hormone concentrations.

4.3.3 Effect of SSTR2 antagonist in the absence of hypoglycemia

All groups that underwent control experiments had similar basal hormone levels (Table 4-4). 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, which suggests that Ctrl:N rats may have been a mildly stressed despite the extreme care taken to avoid stress during handling.

Glycemia remained steady throughout the 4-h experiment (Figure 4-11). Notably, glycemia in D+SSTR2a was unaltered, which demonstrates that SSTR2 antagonist administration did not affect glycemia during basal conditions.

Plasma glucagon levels remained unaltered at all time points in N and D groups during the control experiments. However, Ctrl:D+SSTR2a demonstrated very modest, transient increases in plasma glucagon at 40, 60, and 90 min (Table 4-4). AUC calculations revealed no significant difference in overall glucagon in Ctrl:D and Ctrl:D+SSTR2a in the absence of
hypoglycemia ($P=0.12$). Plasma corticosterone, epinephrine, and norepinephrine levels also remained similar in all groups (Table 4-4). These controls demonstrate that SSTR2 antagonist, *per se*, did not elicit hyperglycemia or marked, sustained elevations of stress hormones.

4.3.4 Pancreatic glucagon and pancreatic and plasma somatostatin measurements

Pancreatic glucagon protein content increased 50% in D compared to N in both hypoglycemia and control studies ($P<0.02$) and was similar in both D and D+SSTR2a (Figure 4-12A). Similarly, pancreatic somatostatin protein content following hypoglycemia was increased by 65% in D rats as compared to N rats ($P<0.02$) and was not affected by SSTR2 antagonist (Figure 4-12B). All three control groups showed no statistical difference in 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: following hypoglycemia, plasma somatostatin increased 60% increase in D, but this failed to reach statistical significance ($P=0.08$, Figure 4-12C).

4.3.5 Liver PEPCK1 and G6Pase mRNA expression

To determine whether the normalization of glucagon and corticosterone responses to hypoglycemia in SSTR2 antagonist-treated diabetic rats also affected the expression of these gluconeogenic enzymes, despite the lack of change in glucose infusion rates, real-time PCR was used to quantitatively measure the levels of mRNA expression in the livers of the high dose insulin group (10 U/kg insulin, 3000 nmol/kg/h SSTR2 antagonist). There was no statistical difference in PEPCK1 expression between all groups, regardless of diabetes, hypoglycemia, and SSTR2 antagonist administration (Table 4-5). In control groups, G6Pase expression was 6-fold lower in Ctrl:D as compared to Ctrl:N ($P<0.0001$). Ctrl:D+SSTR2a showed increased G6Pase expression as compared to Ctrl:D ($P<0.05$) during basal
conditions. Insulin-induced hypoglycemia significantly suppressed G6Pase expression in N ($P<0.0001$) and D+SST2Ra ($P<0.007$).

4.3.6 Effect of SSTR2 antagonist in non-diabetic rats during hypoglycemia

We assessed the effect of SSTR2 antagonist during hypoglycemia in non-diabetic rats. Basal metabolic parameters of these rats (Table 4-6) were similar to non-diabetic controls. Blood glucose levels during hypoglycemia clamp experiments were the same in N and N+SSTR2a at each of the dosing combinations of insulin to induce hypoglycemia and SSTR2 antagonist tested (Figure 4-13A-C). At 1500 nmol/kg/h, SSTR2 antagonist had no effect on glucagon responses to hypoglycemia in non-diabetic rats (Figure 4-14A). At 3000 nmol/kg/h, SSTR2 antagonist had an opposite effect and strikingly decreased plasma glucagon levels in response to hypoglycemia at the 40, 60, 90, and 120 min time points (Figure 4-14B, C). This suppressive effect of 3000 nmol/kg/h SSTR2 antagonist in non-diabetic rats was more marked at the higher dose of insulin (10 U/kg, Figure 4-14B) than at the lower dose of insulin (5 U/kg, Figure 4-14C). SSTR2 antagonism had no effect on corticosterone responses to hypoglycemia in both of the doses tested (Figure 4-14D-F). N+SSTR2a rats had similar pancreatic and plasma somatostatin levels as N following hypoglycemia (Table 4-7).

4.4 Discussion

4.4.1 Role for SSTR2 in the enhancement of glucagon counterregulation

The attenuation or absence of the glucagon response to hypoglycemia in diabetes is well established (165;166). We demonstrate for the first time that antagonism of SSTR2 enhances hypoglycemia-stimulated, but not basal, glucagon and corticosterone release in
diabetic rats. In the present study, administration of SSTR2 antagonist (3000 nmol/kg/h) fully restored glucagon counterregulation to hypoglycemia in diabetic rats induced with either high (10 U/kg) or low (5 U/kg) dose insulin.

The inhibitory effect of somatostatin on α-cell glucagon release via SSTR2 is well-established in both rodents (389;531;532) and humans (385;533). In very early studies, anti-somatostatin antibodies were shown to increase stimulated glucagon release from isolated, non-diabetic islets (279), thus demonstrating that blocking the actions of somatostatin could enhance glucagon secretion. A recent study demonstrated that global somatostatin knockout increased nutrient-stimulated, but not basal, glucagon secretion compared with wild-type mice in vivo and in isolated islets, the latter implicating the profound role of locally released somatostatin on stimulated glucagon secretion (534). In addition, in STZ-treated rats treated with insulin, it was demonstrated that the glucagon response to insulin-induced hypoglycemia can be enhanced by pancreatic infusion and subsequent switch-off (at hypoglycemia) of somatostatin, thereby supporting the notion that glucagon counterregulation can be enhanced by deactivating intrapancreatic signals suppressing α-cells (179). The localization of particular receptor subtypes on different islet cell types allows for specific receptor agonists/antagonist to exert specific effects on the cells. In rodent islets, SSTR2 are found nearly exclusively on glucagon-secreting α-cells while SSTR5 are found on β-cells (388;389). Studies in whole-body somatostatin receptor-type-2 knockout (SSTR2 KO) mice showed that stimulated glucagon secretion was approximately 2-fold greater in islets isolated from SSTR2KO mice than wild-type (389). In humans, somatostatin also exerts its inhibitory effects on glucagon secretion via SSTR2 on α-cells (385;535). An in vitro study in human islets demonstrated that an agonist to the SSTR2 was more potent than other receptor subtype agonists at inhibiting arginine-
stimulated glucagon secretion and that a dose-dependent reversal of SSTR2-agonist-induced glucagon suppression was achieved by using the same SSTR2 antagonist as the present study (386). The findings from this study in human islets suggest that our present work using this SSTR2 antagonist may also be relevant to human studies. Taken together, these studies support the role of somatostatin acting via the SSTR2 in inhibiting stimulated glucagon secretion.

We demonstrate that pancreatic content of glucagon remains elevated in diabetic rats even subsequent to hypoglycemia. This supports our idea that during hypoglycemia, there is a defect in the secretory mechanism of the diabetic α-cell where glucagon remains present, which contributes to impaired glucagon counterregulation. We speculate that this defect is due, at least in part, to increased pancreatic somatostatin and increased insulin sensitivity of diabetic α-cells. In addition, we observe that pancreatic glucagon content is unchanged with SSTR2 antagonist treatment, yet diabetic rats which received SSTR2 antagonist had markedly enhanced glucagon release in response to hypoglycemia. This may suggest that the amount of glucagon secreted in response to hypoglycemia represents only a small amount of glucagon stored in the pancreas or that glucagon is quickly re-synthesized after its secretion in diabetic rats that received SSTR2 antagonist.

Presently, we observe that pancreatic somatostatin remains elevated in diabetic rats following hypoglycemia as previously reported (174). It is because diabetic rats have elevated somatostatin that we decided to double our initial dose of SSTR2 antagonist from our pilot studies. Elevated pancreatic and circulating somatostatin has been reported in various diabetic species, including type 1 diabetic humans, STZ-diabetic and alloxan-diabetic animals, and NOD mice, but BB rats are one diabetic model in which pancreatic somatostatin is not increased (536-539). Untreated diabetic BB rats demonstrated reduced
concentration of pancreatic somatostatin (540). However, circulating somatostatin remains elevated in BB rats (537;541). BB rats also have impaired glucagon and catecholamine responses to hypoglycemia (263;542;543). Thus, increased pancreatic somatostatin is not the only factor for impaired counterregulation. In the present study, there was a trend for increased plasma somatostatin in diabetic groups, although this did not reach statistical significance (Figure 4-12). Interestingly, however, diabetic rats in the control group under basal conditions did not exhibit elevated somatostatin. It has been reported that fasting for 15-h (544) and 48-h (545) decreased pancreatic somatostatin levels in rats. This effect of fasting may be biphasic since with prolonged fasting of 72 h in rats, pancreatic somatostatin levels eventually become elevated (544). At the time of euthanasia when pancreata were harvested, these rats would have been fasted for 24 h. Thus, the reduction in pancreatic somatostatin under basal conditions could be due to a more profound effect of fasting on diabetic groups. When we combined the data within each treatment group for all rats that were subjected to hypoglycemia Table 4-7, we can observe a significant effect of diabetes in elevating both pancreatic ($P<0.0004$) and plasma somatostatin ($P<0.004$) levels. Even if somatostatin is not excessive, suppression of somatostatin may be useful to alleviate further inhibition on diabetic $\alpha$-cells, which are already more sensitive to inhibition by exogenous insulin during hypoglycemia.

Some may not have considered increased somatostatin to be a strong candidate for impaired glucagon responsiveness because somatostatin-secreting $\delta$-cells have been reported to be downstream of glucagon-secreting $\alpha$-cells in the islet microcirculation of non-diabetic rats (79;284). This differed from earlier views that suggested a parallel distribution of $\alpha$-cells and $\delta$-cells in all species and in which $\beta$-$\delta$-$\alpha$ represented the core-to-periphery arrangement of islet cells (276). Since pancreata from non-diabetic rats perfused with
SSTR2 antagonist indeed show increased stimulated glucagon secretion (392), this supports the role of somatostatin in the local regulation of glucagon release via the microcirculation or interstitial interactions. In STZ-diabetic rats, however, this architecture of islet cell type is altered such that δ-cells are also distributed in central portions of islet cells (546). The arrangement of human endocrine islet cells is likewise more disperse throughout the islet (304;547). Furthermore, immunofluorescent methods in pancreata of STZ-diabetic rats, as well type 1 diabetic humans, revealed hyperplasia and hypertrophy of pancreatic δ-cells (439). These findings suggest that paracrine actions of islet hormones may be altered in diabetes such that δ-cell release of somatostatin may very well regulate glucagon secretion.

4.4.2 Potential role for SSTR2 in insulin secretion?

In some studies in human perfused pancreas (548) and of incubations of cloned hamster β-cell lines (549), it was demonstrated that activation of SSTR2 could inhibit insulin secretion, which may lead to speculation that SSTR2 antagonism may promote insulin release. The laboratory of Efendic also demonstrated a moderate (≈30%) increase in arginine-stimulated insulin secretion with the same SSTR2 antagonist (PRL-2903) in batch incubated islets of non-diabetic rats (392). It should be noted, however, that the increase in glucagon secretion (5-fold) under the same conditions was much greater thus demonstrating a much larger effect of the SSTR2 antagonist on stimulated glucagon, rather than insulin, secretion. There was no effect of the SSTR2 antagonist in insulin secretion in perifused isolated islets or perfused pancreas. Although this SSTR2 antagonist has a 20-fold greater binding affinity for SSTR2 than SSTR5 (expressed on rodent β-cells), high concentrations of SSTR2 antagonist may exert a non-specific effect if sufficient amounts of a native competitor (i.e. endogenous somatostatin) are not present. This concept of non-specific role of SSTR2 in promoting insulin secretion will be discussed further in section 4.4.9 in the interpretation of
our data for SSTR2 antagonism in non-diabetic rats during hypoglycemia. In contrast, our experiments conducted under basal conditions do not demonstrate an effect on insulin release with SSTR2 antagonism, presumably primarily because of STZ-diabetic rats have markedly less pancreatic insulin due to β-cell destruction, and also because SSTR5 rather than SSTR2 is the predominant isoform of SSTR on rodent β-cells (387). It is possible that SSTR2 antagonism may have some effect on insulin secretion in humans since some SSTR2 are detected human, but not rodent, β-cells, (385). This expression is relatively low as it was reported in pancreatic islets of non-diabetic humans that approximately 45% of β-cells expressed SSTR2 whereas approximately 100% and 90% expressed SSTR1 and SSTR5, respectively. Furthermore, SSTR2 antagonism is unlikely to exacerbate hypoglycemia if used clinically in diabetic humans since individuals with type 1 diabetes will not have functioning insulin-secreting β-cells.

4.4.3 Role for SSTR2 in the enhancement of corticosterone counterregulation

Whereas glucagon is important during the early fall in plasma glucose, catecholamines and glucocorticoids become increasingly important at lower glucose levels and during prolonged hypoglycemia (3;232). Dysregulation of the hypothalamo-pituitary-adrenal (HPA) axis can contribute to defective counterregulation, since it not only regulates glucocorticoid secretion but also affects epinephrine secretion (202;220;550-554). Glucocorticoids increase liver sensitivity to glucagon and epinephrine as well as decrease hepatic and peripheral insulin sensitivity (555;556). Presently, we demonstrate that peripheral administration of SSTR2 antagonist normalizes the attenuated corticosterone response to hypoglycemia in diabetic rats, which may be due to a central effect of somatostatin antagonism or to a peripheral effect of SSTR2 antagonism at the level of the pituitary or adrenal glands. The attenuated corticosterone response in diabetic rats occurred in both high (10 U/kg) and low (5 U/kg)
dose insulin-induced hypoglycemia experiments in which basal corticosterone was either
elevated or similar to non-diabetic rats. The markedly blunted corticosterone responses of
diabetic rats as compared with non-diabetic supports previous findings (202;205). In our
low-dose insulin-induced hypoglycemia experiments, plasma insulin levels were similar at all
time after insulin administration. Despite the fact that all rats in the high dose insulin
experiments received 10 U/kg, plasma insulin levels at t=60 and 180 min were
approximately 50% elevated in non-diabetic as compared to diabetic controls. This may be
due to enhanced clearance of exogenous insulin by diabetic rats. It has been reported that
insulin dose and consequent plasma levels, per se, affect hormonal responses to
hypoglycemia. In non-diabetic humans, a 6-fold higher insulin level significantly increased
cortisol and catecholamine, but not glucagon, responses by 58% and 33%, respectively, to
hypoglycemia despite an equivalent level of hypoglycemia, but this amplifying effect of
insulin was lost in subjects with type 1 diabetes (209;210). Thus, the higher plasma insulin
levels in our non-diabetic rats of the high-dose insulin-induced hypoglycemia study may be
of concern. In our studies, high-dose insulin yielded a 2-fold greater peak plasma insulin
level as compared to low-dose insulin experiments in our non-diabetic rats; however, peak
cortisol, epinephrine, and norepinephrine levels were similar. Glucagon responses were
slightly, but not significantly, greater in high-dose insulin as compared with low-dose insulin
regimes. Thus, in our studies, it is unlikely that insulin, per se, caused an amplifying effect
of counterregulatory hormones to hypoglycemia, and thus the difference in peak plasma
insulin levels between non-diabetic and diabetic controls in the high-dose insulin
experiments would not account for the observation of attenuated corticosterone (or
glucagon) counterregulation in diabetic rats.
Basal corticosterone levels are often reported to be elevated in diabetic rats, an effect which is attributed to increased HPA activity due to molecular HPA dysregulation (202;205;557). Increased basal corticosterone in diabetes was observed in our high dose insulin hypoglycemia experiments, but not in our low dose insulin hypoglycemia or control experiments. We attribute the lack of elevated corticosterone to increased basal corticosterone levels of our non-diabetic controls since these values were augmented approximately 4-fold relative to our non-diabetic rats in the high dose insulin group, which had a comparatively larger sample size. This increase in basal corticosterone in non-diabetic rats may have been due to unwanted stress effects, but since basal epinephrine levels remained low, any unwanted stress was likely very mild. A circadian effect is also possible if basal hormone samples of thee non-diabetics were obtained slightly later in the day. It is known that in the non-diabetic rat, a nocturnal animal, the corticosterone nadir occurs in the morning and peaks in the evening (558). However, diabetic rats (559), as well as humans (560), disrupted circadian patterns of corticosterone/cortisol secretion, with elevated levels during the trough and normal or slightly elevated values during peak secretion. However, this is unlikely to have affected our results since the patterns of corticosterone responses to hypoglycemia were similar in both sets of hypoglycemia experiments.

The role of somatostatin on the regulation of corticosterone is less well-documented than its role on suppressing glucagon release. However, somatostatin also inhibits the secretion of both CRH and ACTH (271;294), which regulate corticosterone secretion; thus, somatostatin inhibits the function of the HPA axis. SSTR2 have been localized in several brain regions and specifically in areas pertaining to HPA activity – the hypothalamic paraventricular nucleus (420) and ACTH cells in the anterior pituitary (561). It is unknown whether this
antagonist crosses the blood-brain-barrier. However, a cyclic octapeptide with a similar chemical structure, CTAP, is known to enter the brain when administered peripherally (562). The corticosterone response to hypoglycemia is often impaired in diabetes (202;205;206;225;563). Our laboratory has previously shown that carbachol injected intracerebroventricularly into the third ventricle (a model of stress) increases the release of cortisol in non-diabetic and diabetic dogs (169). However, when somatostatin was given acutely with carbachol, cortisol responses were abolished in both normal and diabetic dogs (528). Furthermore, somatostatin has been demonstrated to inhibit arginine vasopressin release in response to hypoglycemia (564). Arginine vasopressin is one of the neuropeptides involved in the activation of the HPA axis during stress. This supports the idea that the SSTR2 antagonist could improve counterregulation through a central pathway and could be a mechanism whereby the SSTR2 antagonist also markedly increases the corticosterone response to hypoglycemia in diabetic rats. Along with other subtypes, SSTR2 are normally expressed in the pituitary (561). Pituitaries of SSTR2 knockout mice release more ACTH in vitro (565), and corticosterone levels are elevated in SST knockout mice (566), which supports the role of endogenous somatostatin on regulating corticosterone release. An alternative possibility is an enhancement of corticosterone through somatostatin receptors in the adrenal cortex. In the normal adrenal gland, all five subtypes of SSTR are identified although SSTR2 and SSTR4 are most highly expressed (567;568). However, despite consistent detection of SSTR2 in the zona glomerulosa (390;397-399), its detection is not as consistent in the corticosterone-synthesizing fasciculata and reticularis zonae of the adrenal cortex (390;569).
4.4.4 Epinephrine responses to hypoglycemia were unaffected

In the present study, we observed no difference in the epinephrine response to hypoglycemia in non-diabetic and three-week STZ-diabetic rats. Although this lack of an epinephrine defect contrasted with our prior experiments in which intravenous insulin infusion was used to induce hypoglycemia (202;205), it is consistent with other observations in which hypoglycemia was induced with a subcutaneous insulin injection (174), suggesting that the route of insulin administration may also play a role in the epinephrine response. An effect of the SSTR2 antagonist on epinephrine levels in diabetic rats was not observed, either because somatostatin has no role in epinephrine counterregulation to hypoglycemia or because the maximal physiological epinephrine response was already achieved. It would be of future interest to evaluate the effect of SSTR2 antagonism in a diabetic model in which a defect of epinephrine is observable (e.g. diabetes of longer duration (570)) to determine whether somatostatin may also play a role in hypoglycemia-stimulated epinephrine release.

Somatostatin inhibits glucagon secretion in response to various stimuli, and it also blocks the effect of epinephrine to stimulate glucagon release as was demonstrated by the exogenous administration of somatostatin to isolated perfused rat pancreas (571). Somatostatin and epinephrine share common mechanistic pathways by which they regulate glucagon release from the α-cell. Somatostatin activation of SSTR2 inhibits adenylate cyclase activity, thus reducing cAMP levels and subsequently inhibits protein kinase A (PKA)-stimulated glucagon release (572-574). PKA is a serine/threonine kinase that phosphorylates substrate proteins at serine or threonine sites (575). In contrast, epinephrine activation of α1- and β-adrenergic receptors on α-cells stimulates adenylate cyclase activity, in turn increasing cAMP levels and promoting PKA-stimulated glucagon
release (576-578). Somatostatin abolishes L-type Ca\textsuperscript{2+} channel-dependent release of glucagon (579), whereas epinephrine enhances Ca\textsuperscript{2+} influx through L-type Ca\textsuperscript{2+} channels which increased intracellular Ca\textsuperscript{2+} levels to promote glucagon release (577). Somatostatin activates calcineurin, a serine/threonine phosphatase that “deprimes” glucagon-containing granules and thus inhibits glucagon release (579), whereas epinephrine accelerates the mobilization of glucagon-containing granules, increasing the number of readily releasable granules by 5-fold (577). Thus, epinephrine can help to enhance glucagon secretion during hypoglycemia, but excessive somatostatin may hinder this enhancement. In our present studies, a robust glucagon response to hypoglycemia in our non-diabetic rats was accompanied with a robust epinephrine response. In our diabetic rats, however, the glucagon response to hypoglycemia was markedly abolished despite epinephrine responses of the same magnitude as non-diabetic rats. When diabetic rats were treated with SSTR2 antagonist, glucagon responses to hypoglycemia were normalized. Thus, we presently hypothesize that SSTR2 antagonism may also alleviate the suppressive effects of somatostatin on the potentiating effects of epinephrine on glucagon release in response to hypoglycemia.

4.4.5 **SSTR2 antagonist demonstrated no effect on growth hormone**

Presently, we see no effect of SSTR2 antagonism on growth hormone release during the 1-h basal period or during 3-h hypoglycemia when SSTR2 antagonist was given at high dose. Furthermore, no increase of growth hormone is observed in response to hypoglycemia. Since growth hormone is known to play a role in prolonged hypoglycemia (143;580), the present hypoglycemia experiments may not have been of sufficient duration to stimulate a growth hormone response. Another possibility for the lack growth hormone increase is that plasma epinephrine levels were markedly increased during hypoglycemia. This elevation of
epinephrine could have suppressed growth hormone, as could an epinephrine-induced increase in free fatty acid levels, both of which are known to suppress growth hormone secretion (581). Also, it should be noted that samples were obtained at very few time points (four time points over a four hour experiment). Since growth hormone release is pulsatile (581), frequent blood sampling is required to accurately measure growth hormone response. Thus, the growth hormone data obtained in the present study may not truly be indicative of the pattern anticipated in response to hypoglycemia. In response to prolonged hypoglycemia, growth hormone counterregulation can contribute to the promotion of glycemic recovery by suppressing insulin-mediated glucose utilization and increasing glucose release into the circulation (582;583). When growth hormone secretion is inhibited, liver and peripheral insulin sensitivity decreases (500;584), and nonesterified fatty acid levels increase (500). SSTR5 is the predominant isoform of SSTR expressed in rat pituitary, followed by the expression of SSTR2. Immunohistochemical colocalization studies demonstrated that SSTR5 is expressed in 86% of rat GH-positive cells as compared to SSTR2 in 42% of rat somatotrophes (379). Furthermore, it has demonstrated that for somatostatin to effectively inhibit growth hormone secretion, the SSTR5 must also be involved (431), and heterodimerization and activation of both SSTR2 and SSTR5 results in synergistic GH suppression (432;585). Thus, it is not surprising that our selective SSTR2 antagonist, which does not have a high affinity for SSTR5, does not affect growth hormone release in our experiments. However, it should be noted that growth hormone is very pulsatile in its release and that we took measurements at relatively few time points and may have missed periods of plasma differences in growth hormone secretion.
4.4.6 **SSTR2 antagonism does not cause hyperglycemia or undesired stress**

We also demonstrate that SSTR2 antagonist does not affect glycemia or markedly alter counterregulatory hormones in the absence of hypoglycemia. The control experiments in the absence of insulin were performed to examine the effects of SSTR2 antagonist, *per se*, during basal conditions.

Somatostatin analogues were clinically used to help lower blood glucose levels in diabetic patients (586;587). Also, the anti-diabetic activity of a SSTR2 agonist was demonstrated in rodent models of type 2 diabetes in which the agonist lowered plasma glucagon and glucose levels (588). Thus, it was important to evaluate the effect of the SSTR2 antagonist when administered during the basal state if SSTR2 antagonism could have any putative clinical role as a therapy for hypoglycemia in the diabetic population. Since basal hyperglucagonemia can persist despite elevated plasma somatostatin levels in diabetes, the role of somatostatin-induced glucagon inhibition has been questioned (489). Presumably, some could speculate that the SSTR2 antagonist may promote basal hyperglycemia, which is undesired in diabetic patients. However, this can be reconciled with understanding an action of somatostatin receptor-coupled signalling in the α-cell: somatostatin via SSTR2 inhibits Ca\(^{2+}\)-induced glucagon exocytosis via G\(_{i2}\)-dependent activation of calcineurin, a serine/threonine protein phosphatase which “deprimes” glucagon-containing granules associated with \(L\)-type Ca\(^{2+}\) channels but does not affect the granules associated with \(N\)-type Ca\(^{2+}\) channels, which mediate basal glucagon secretion (577;579;589). In addition to this mechanism, somatostatin can also inhibit adenylate cyclase activity, consequently reducing cAMP levels and protein kinase A (PKA)-stimulated glucagon secretion, as well as activate G-protein-coupled K\(^{+}\) channels on α-cells, which causes hyperpolarization of the
plasma membrane and subsequent inhibition of α-cell electrical activity and glucagon release (589). 

Presently, we demonstrate that antagonism of SSTR2 enhances hypoglycemia-stimulated, but not basal, glucagon release in diabetic rats. In vitro experiments in pancreatic islets of non-diabetic mice demonstrate that blocking somatostatin receptor type 2 using SSTR2 antagonist stimulated glucagon secretion in low glucose conditions but did not affect glucagon secretion at “normal” glucose conditions of 7 mM (590). Administration of SSTR2 antagonist in perfused non-diabetic rat pancreas did not affect the inhibitory effect of hyperglycemia on glucagon secretion (591), thus confirming that SSTR2 did not elicit hyperglucagonemia when glucose concentrations were already high. Presently, we demonstrate that SSTR2 antagonism during basal conditions in vivo yielded very small transient increases of glucagon which did not cause hyperglycemia and did not alter corticosterone or catecholamine levels, suggesting that this antagonist does not elicit marked, non-stimulated secretion of counterregulatory hormones. However, it remains to be investigated whether prolonged administration of SSTR2 antagonists will lead to hyperglycemia. Studies in SSTR2 knockout mice showed no differences in fasting and fed blood glucose levels, fasting and fed plasma glucagon levels, or responses to glucose tolerance tests compared wild-type controls, but diet-induced obesity in these knockouts caused fasting and fed hyperglycemia, fed hyperglucagonemia, and impaired glucose tolerance compared to wild-types given the same high-fat diet (592). It remains to be seen if a prolonged administration of SSTR2 antagonist in diabetic animals would exacerbate diabetes.
4.4.7  **Glucose requirement during hypoglycemia**

Glucose infusion rates and total glucose infused were decreased with SSTR2 antagonism during low-dose insulin-induced hypoglycemia in the present study. A lower glucose requirement during hypoglycemia is consistent with normalized glucagon and corticosterone responses in the SSTR2 antagonist-treated diabetic group.

At the high insulin dose, there were no differences in glucose infusions rates between non-diabetic and diabetic rats. With three weeks of chronic hyperglycemia in our STZ-diabetic rats, it would be anticipated that some insulin resistance would develop, as has been previously reported (205). However, glucose infusion rates were not lower in untreated diabetic rats during hypoglycemia presumably because the expected insulin resistance of diabetic rats was offset by the generally lower counterregulatory response. Furthermore, the hypothesized reduction in glucose requirement in SSTR2 antagonist treated animals may have been masked by the overwhelming maximal inhibitory effect of insulin to suppress endogenous glucose production and increase glucose utilization. Our method of inducing hypoglycemia with insulin injection creates a major perturbation of steady state which renders classic glucose turnover techniques to measure glucose production and utilization less reliable, especially during the rapid change of glycemia at the onset of insulin injection in the present experiments, thus we used the exogenous glucose requirement and expression of gluconeogenic enzymes as indicators of effects of improved counterregulation on glucose homeostasis.

Despite using half the dose of insulin in our low-dose versus high-dose insulin experiments, glucose infusion rates seemingly appear greater in groups induced with 5 U/kg insulin. We attribute this to the fact that hypoglycemia was reached at an earlier time point in the low-dose study (i.e. non-diabetic rats: at t=20 min for low-dose as compared to t=50 min for
high dose insulin-induced hypoglycemia experiments; both diabetic groups: at t=25 min for low-dose as compared to t=70 min for high-dose insulin-induced hypoglycemia experiments), and thus the glucose requirement would be greater to maintain clamped hypoglycemia for a longer duration. Alternatively, diabetic rats in the low-dose insulin experiments may have had a greater exogenous glucose requirement since they had a lower initial basal glycemia and may have been less insulin resistant than diabetic rats in the high-dose group. Regardless, diabetic groups within each experiment were similar, and thus the effects of SSTR2 antagonist treatment could be accurately compared.

4.4.8 Expression of gluconeogenic enzymes

Gluconeogenesis plays a role in restoring blood glucose levels in response to hypoglycemia and is particularly relevant after an overnight fast in which glycogen stores are depleted in rodents, resulting in gluconeogenesis rather than glycogenolysis as the main contributor to glucose synthesis (593-595). Glucagon (596-598) and corticosterone (599-601) are well known to induce the expression of PEPCK and G6Pase. Glucagon activates protein kinase A, increasing cAMP levels, which induces gluconeogenic enzymes (596-598). Corticosterone induces PEPCK (599) and G6Pase (600;601) expression by binding with glucocorticoid receptors and activating glucocorticoid-responsive elements on the promoter regions of these genes. Furthermore, the nuclear coactivator protein peroxisome proliferator activated receptor-γ coactivator 1α (PGC-1α) is induced by glucagon and corticosterone and promotes increased transcription of both PEPCK and G6Pase by its interaction with HNF-4α, thus contributing as a key mechanism for controlling glucose production in hepatocytes (602). Since glucagon and corticosterone responses to hypoglycemia were normalized in diabetic rats with SSTR2 antagonism without an effect on glucose infusion rates at the high insulin dose, we wished to determine if gene expression of these gluconeogenic enzymes was
increased and this would have served as better markers than glucose infusion rates of counterregulatory hormone actions.

Hypoglycemia markedly decreased G6Pase mRNA expression in both non-diabetic and diabetic rats, which is attributed to the large amount of insulin administered (up 200-fold increase in plasma levels), which has a large suppressive effect on gluconeogenic enzyme synthesis (596;603) and can override the stimulatory effect of increased counterregulatory hormones (604). PEPCK1, the cytosolic form, is the predominant form in rodent liver (605). Unlike G6Pase, expression of PEPCK1 was not decreased by hypoglycemia in the present study. This is in accordance with another study in which epinephrine could overcome the inhibitory effect of insulin on stimulating PEPCK1 expression in rat hepatocytes (606). Thus, despite robust increases of glucagon and corticosterone during hypoglycemia, the expression of PEPCK1 and G6Pase was not affected, perhaps masked by the stimulatory effect of epinephrine or overwhelming inhibitory effect of insulin, respectively.

In the absence of hypoglycemia, G6Pase in diabetic rats was actually decreased compared with non-diabetic rats, and SSTR2 antagonist treatment in diabetic rats partially increased this expression. The modest, transient increase of plasma glucagon levels in the SSTR2 antagonist-treated group may be responsible for the moderate increase in G6Pase expression in the present study. Modest increases in circulating glucagon are sufficient in increasing glucose production in the short-term (i.e. 2 h) in partially insulin-deprived humans (607), although in our study, hyperglycemia was not observed during the 4-h time-frame of our experiments. Our basal gluconeogenic enzyme results are in contrast to the popular view that expression of gluconeogenic enzymes PEPCK (608-610) and G6Pase (601;611-614) are elevated with diabetic rodents, but are in line with a recent finding in mildly hyperglycemic, overnight fasted, STZ-diabetic rats, (615). A shorter duration of
fasting (i.e. 6-h) in STZ-diabetic rats likewise yielded no differences in PEPCK1 and GP6Pase expression despite elevated fasting plasma glucose levels and rates of endogenous glucose production (615). In addition to suggesting the role of other key enzymes which may be responsible for regulating gluconeogenesis, the authors suggest that increased PEPCK and G6Pase expression are often associated with diabetic models with increased corticosterone levels and demonstrate that expression does not increase from controls in STZ-diabetic rats without hypercorticosteronemia. In our non-diabetic and diabetic rats which had similar plasma corticosterone levels for the duration of the control experiments, we likewise do not observe increased expression of gluconeogenic enzymes. Another possibility is that basal gluconeogenic enzyme transcription is not increased in our diabetic rats despite lower endogenous insulin because of hyperglycemia. At present, there is no known direct effect of SSTR2 antagonism on hepatic gluconeogenesis. SSTR1 (271) and SSTR3 (373), but not SSTR2, have been detected on hepatocytes, and the SSTR2 antagonist has no reactivity with SSTR1 and 10-fold less binding affinity with SSTR3 (509). In isolated rat hepatocytes, somatostatin has been reported to have no direct effects on basal glucose production (616;617) on glucagon-stimulated glucose production, or on PEPCK and G6Pase activity (617). Similarly, studies in conscious dogs have demonstrated that somatostatin does not alter basal glucose production rates when the levels of insulin and glucagon are maintained (618). Besides its use in pancreatic clamp studies to measure glucose turnover, the direct effects of somatostatin effect on the liver are not well-documented aside from its use in liver tumour cells or cirrhotic liver (418). Somatostatin bound to its receptors elicit their cellular responses through G-protein coupling, including inhibition of adenylyl cyclase via inhibitory G proteins (271), thus it is possible that somatostatin can lower cAMP levels directly within hepatocytes, consequently blunting expression of gluconeogenic enzymes such as PEPCK and G6Pase and thus affecting gluconeogenesis and subsequent hepatic glucose production.
However, there is no evidence that such a mechanism exists at the present time. However, the modest, transient increase of plasma glucagon levels in the SSTR2 antagonist-treated group may be responsible for the moderate increase in G6Pase expression observed in the present study.

4.4.9 Effect of SSTR2 antagonism during hypoglycemia in non-diabetic rats

The main objective of this study was to evaluate the effect of SSTR2 blockade on hormone counterregulation to hypoglycemia in diabetic rats. We postulated that elevated somatostatin in diabetes contributes to this impairment, and SSTR2 antagonism in non-diabetic rats provides a control during hypoglycemia so that the magnitude of the impairment in diabetes that might be attributable to elevated somatostatin may be assessed. Both groups of non-diabetic rats in this study have similar pancreatic and plasma somatostatin levels (Table 4-7). We presently provide evidence that SSTR2 antagonist treatment in non-diabetic rats does not increase glucagon or corticosterone responses to hypoglycemia. This is in contrast to diabetic rats with elevated somatostatin in which disinhibition of somatostatin acting via SSTR2 normalized the defects of both glucagon and corticosterone counterregulation. We assessed the effects of diabetes and administration of SSTR2 antagonist on pancreatic and plasma somatostatin levels by combining available data of the groups form the hypoglycemia experiments (Table 4-7). Using Two-Way ANOVA statistical analysis, it can be shown that there is an effect of diabetes, but not SSTR2 antagonist \((P=0.51 \text{ for both})\), on both pancreatic somatostatin \((P<0.0004)\) and plasma somatostatin \((P<0.004)\) values. Thus, data from the non-diabetic control for SSTR2 antagonism during hypoglycemia provides support for a role of elevated somatostatin in diminishing glucagon and corticosterone responses to hypoglycemia in diabetic rats, but not non-diabetic rats.
At 1500 nmol/kg/h, SSTR2 antagonist had no effect on glucagon responses to hypoglycemia in non-diabetic rats. This supports the hypothesis that somatostatin does not play a role in suppressing glucagon release in non-diabetic rats, but taken together with the data in diabetic rats in which somatostatin is elevated, it demonstrates that disinhibition of the α-cell (which is has increased sensitivity to the suppressive effects of exogenous insulin) during hypoglycemia enhances glucagon release in diabetes. However, it was very surprising to observe that when a two-fold larger dose of SSTR2 antagonist was used in non-diabetic rats, an effect opposite to that which would be expected of somatostatin blockade ensued: glucagon responses were markedly attenuated rather than enhanced. This effect could have been mediated by a stimulated release of insulin resulting from a non-specific effect of a high dose SSTR2 antagonist on β-cells (which express SSTR5 and not SSTR2 in rats), which could locally act to suppress glucagon release. It is possible that when high doses of this antagonist are used in the absence of a sufficient amount of a competitor (i.e. non-diabetic rats have significantly less somatostatin than diabetic rats), binding to the SSTR5 on β-cells may occur, which could disinhibit the β-cell from the suppressive effects of somatostatin and promote insulin secretion. Cejvan et al. reported that this SSTR2 antagonist under certain conditions at high dose modestly increased insulin secretion in non-diabetic rat islets when it should have had no effect (392). A similar concept has previously been suggested by Gromada et al. (589) who used a high dose of SSTR2 antagonist in studies of isolated rat β-cells. In this in vitro setting in which the purified β-cell fraction did not contain any detectable amounts of endogenous somatostatin, the SSTR2 antagonist applied at a comparatively high dose (2 μM) increased insulin secretion, which led to the authors cautioning against the use of these compounds in the absence of native competitive receptor agonists (589). Thus, despite the fact that our SSTR2 antagonist binds with 20-fold lower affinity to SSTR5 than SSTR2, thus suppressing
glucagon release, it is possible that β-cell release of insulin may have been stimulated by high dose SSTR2 antagonist in non-diabetic rats of our study. Non-diabetic rats did not have augmented pancreatic somatostatin as diabetic rats, and the excessive amount of the peptide may have suppressed the α-cell rather than disinhibiting it. Future analysis using a C-peptide assay could determine whether SSTR2 antagonist stimulates insulin release in non-diabetic rats. This would not occur in diabetic rats, which have both elevated somatostatin and markedly reduced β-cell function. Alternatively, it is also possible that at high doses, our peptide antagonist could have partial agonist activity. One group has cautioned that certain competitive peptide analogues could exert opposite effects when used at higher concentrations (619).

4.5 Study 1 Conclusions
In conclusion, we demonstrate that glucagon and corticosterone counterregulatory hormone responses to hypoglycemia can be normalized in diabetic rats with specific antagonism of the SSTR2, which does not elicit hyperglycemia or substantial elevations in counterregulatory hormones during basal conditions. Our results therefore suggest an important role for increased pancreatic, and possibly circulating, somatostatin in defective glucagon and corticosterone counterregulation in diabetes but do not rule out other mechanisms of impairment. Under conditions of lower dose insulin injection, SSTR2 antagonism reduced the glucose requirement during hypoglycemic clamp, which is indicative of a lower risk of hypoglycemia. In this model of diabetes, epinephrine and norepinephrine counterregulation to hypoglycemia induced with both high and low doses of insulin were similar in both non-diabetic and diabetic rats. Growth hormone levels were not affected by SSTR2 administration.
Figure 4-1. Study 1 protocol design.

day -7 to 0:
- acclimatization of male Sprague-Dawley rats (300-400 g)

day 0:
- sham injection (ip saline) OR induction of diabetes (ip STZ (65 mg/kg))

day 13:
- catheterization surgery (L carotid artery & R jugular vein)

day 20:
- overnight fast (16-18 h)

day 21:
- Non-diabetic (N)
- Diabetic (D)
- Diabetic+SSTR2a antagonist (D+SSTR2a)

Pilot:
- 10 U/kg insulin
- 1500 nmol/kg/h SSTR2a
- N+SSTR2a: n=3

- 10 U/kg insulin
- 3000 nmol/kg/h SSTR2a
- N+SSTR2a: n=4

- 5 U/kg insulin
- 3000 nmol/kg/h SSTR2a
- N+SSTR2a: n=10

Non-diabetic+SSTR2a antagonist (N+SSTR2a)

Hypoglycemia Experiments

Control Experiments
(SSTR2a in the absence of hypoglycemia)
Figure 4-2. Schematic representation of A) hypoglycemia clamp experiments (solid lines) and B) control experiments during basal conditions in the absence of exogenously administered insulin (dashed lines). In (A), “x” represents dose of insulin (high dose = 10 U/kg, low dose = 5 U/kg) and “y” represents dose of SSTR2 antagonist (pilot study = 1500 nmol/kg/h, else = 3000 nmol/kg/h). In (B), no insulin or glucose infusions are administered in these experiments under basal conditions. White diamonds (◊): N; black squares (■): D; grey circles (★): D+SSTR2a.
Figure 4-3. Pilot study: Plasma hormone levels during hypoglycemia clamp (10 U/kg insulin, 1500 nmol/kg/h SSTR2 antagonist). A) Glucagon. B) Corticosterone. White diamonds (◊): N; black squares (■): D; grey circles (●): D+SSTR2a. White bars: N; black bars: D, grey bars: D+SSTR2a. Data are represented as means ± SEM. *P<0.05 D vs N. †P<0.05 D vs D+SSTR2a.
Figure 4-4. Blood glucose levels during hypoglycemia clamp (10 U/kg insulin, 3000 nmol/kg SSTR2 antagonist). White diamonds (◊): N; black squares (■): D; grey circles (♦): D+SSTR2a. Data are represented as means ± SEM.
Figure 4-5. Plasma hormone levels during hypoglycemia clamp (10 U/kg insulin, 3000 nmol/kg/h SSTR2 antagonist). A) Glucagon. B) Corticosterone. C) Glucose infusion. White diamonds (◊): N; black squares (■): D; grey circles (○): D+SSTR2a. White bars: N; black bars: D, grey bars: D+SSTR2a. Data are represented as means ± SEM. *P<0.002 D vs N. †P<0.05 D vs D+SSTR2a.
Figure 4-6. Plasma A) epinephrine and B) norepinephrine levels during hypoglycemia clamp (10 U/kg insulin, 3000 nmol/kg/h SSTR2 antagonist). White diamonds (◊): N; black squares (■): D; grey circles (●): D+SSTR2a. Data are represented as means ± SEM.
Figure 4-7. Blood glucose levels during hypoglycemia clamp using low insulin dose (5 U/kg insulin, 3000 nmol/kg SSTR2 antagonist). White diamonds (◊): N; black squares (■): D; grey circles (⊙): D+SSTR2a. Data are represented as means ± SEM.
**Figure 4-8.** Plasma hormone levels during hypoglycemia clamp (5 U/kg insulin, 3000 nmol/kg/h SSTR2 antagonist).  

**A)** Glucagon.  
**B)** Corticosterone.  
**C)** Glucose infusion.  
White diamonds (◊): N; black squares (■): D; grey circles (●): D+SSTR2a.  White bars: N; black bars: D, grey bars: D+SSTR2a.  Data are represented as means ± SEM.  *P<0.05 D vs N.  †P<0.05 D vs D+SSTR2a.
Figure 4-9. Plasma A) epinephrine and B) norepinephrine levels during hypoglycemia clamp (5 U/kg insulin, 3000 nmol/kg/h SSTR2 antagonist). White diamonds (◊): N; black squares (■): D; grey circles (●): D+SSTR2a. Data are represented as means ± SEM.
**Figure 4-10.** Plasma growth hormone levels during hypoglycemia clamp (5 U/kg insulin, 3000 nmol/kg/h SSTR2 antagonist). White diamonds (◊): N; black squares (■): D; grey circles (●): D+SSTR2a. Data are represented as means ± SEM.
**Figure 4-11.** Blood glucose levels during control experiments in the absence of insulin (0 U/kg, 3000 nmol/kg/h). White diamonds (◊): N; black squares (■): D; grey circles (○): D+SSTR2a. Data are represented as means ± SEM.
Figure 4-12. Pancreatic protein content of A) glucagon and B) somatostatin, and C) plasma somatostatin, following hypoglycemia clamp (10 U/kg insulin, 3000 nmol/kg/h SSTR2 antagonist) and control experiments in the absence of insulin (0 U/kg, 3000 nmol/kg/h). Solid bars represent hypoglycemia experiments. Hashed bars represent control experiments. White bars: N; black bars: D, grey bars: D+SSTR2a. Data are represented as means ± SEM. *P<0.02 D vs N.
Figure 4-13. Blood glucose levels during hypoglycemia clamp in non-diabetic rats at the various dosing combinations of insulin and SSTR2 antagonist. A) 10 U/kg insulin, 1500 nmol/kg/h SSTR2 antagonist; B) 10 U/kg insulin 3000 nmol/kg/h SSTR2 antagonist; (C) 5 U/kg insulin, 3000 nmol/kg/h SSTR2 antagonist. White diamonds (◊): N; grey triangles (▲): N+SSTR2a. Data are represented as means ± SEM.
Figure 4-14. Effect of SSTR2 antagonist on plasma glucagon A-C) and corticosterone D-F) at the various dosing combinations of insulin and SSTR2 antagonist in non-diabetic rats. 

**TOP (A, D):** 10 U/kg insulin, 1500 nmol/kg/h SSTR2 antagonist; **MIDDLE (B, E):** 10 U/kg insulin 3000 nmol/kg/h SSTR2 antagonist; **BOTTOM (C, F):** 5 U/kg insulin, 3000 nmol/kg/h SSTR2 antagonist. White diamonds (◇): N; grey triangles (▲): N+SSTR2a. Data are represented as means ± SEM. *P<0.05 N vs N+SSTR2a. †P<0.005 N vs. N+SSTR2a.
Table 4-1. Metabolic parameters of rats that underwent hypoglycemia clamps or control experiments in the absence of insulin. Fed blood glucose, body weight, and food intake were measured daily, and the table below shows the mean of values averaged over the last week before the experimental day. Daily blood glucose measurements were obtained using a glucometer. Data are represented as means ± SEM. *P<0.0002 D vs N in the respective experiment. **P<0.03 D vs N in the respective experiment. †P<0.04 D vs D+SSTR2a in the respective experiment.

<table>
<thead>
<tr>
<th>(pilot study) hypoglycemia clamps (10 U/kg; 1500 nmol/kg/h)</th>
<th>fed blood glucose (mM)</th>
<th>body weight (g)</th>
<th>food intake (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N (n=5)</td>
<td>5.3 ± 0.2</td>
<td>408 ± 5</td>
<td>18 ± 2</td>
</tr>
<tr>
<td>D (n=3)</td>
<td>28.1 ± 1.8 *</td>
<td>340 ± 7 *</td>
<td>42 ± 3 *</td>
</tr>
<tr>
<td>D+SSTR2a (n=3)</td>
<td>26.3 ± 2.7</td>
<td>351 ± 9</td>
<td>36 ± 3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>hypoglycemia clamps (10 U/kg; 3000 nmol/kg/h)</th>
<th></th>
<th></th>
<th></th>
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<tbody>
<tr>
<td>N (n=14)</td>
<td>5.2 ± 0.1</td>
<td>435 ± 7</td>
<td>23 ± 1</td>
</tr>
<tr>
<td>D (n=14)</td>
<td>22.4 ± 1.2 *</td>
<td>365 ± 5 *</td>
<td>37 ± 2 *</td>
</tr>
<tr>
<td>D+SSTR2a (n=18)</td>
<td>22.8 ± 1.1</td>
<td>355 ± 6</td>
<td>30 ± 1 †</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>hypoglycemia clamps (5 U/kg; 3000 nmol/kg/h)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>N (n=5)</td>
<td>5.6 ± 0.3</td>
<td>400 ± 15</td>
<td>19 ± 3</td>
</tr>
<tr>
<td>D (n=4)</td>
<td>24.8 ± 3.5 *</td>
<td>323 ± 20 **</td>
<td>32 ± 4 **</td>
</tr>
<tr>
<td>D+SSTR2a (n=4)</td>
<td>25.6 ± 1.0</td>
<td>331 ± 25</td>
<td>35 ± 3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>controls in absence of insulin (0 U/kg; 3000 nmol/kg/h)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Ctrl:N (n=7)</td>
<td>5.8 ± 0.1</td>
<td>419 ± 6</td>
<td>18 ± 1</td>
</tr>
<tr>
<td>Ctrl:D (n=7)</td>
<td>24.7 ± 1.3 *</td>
<td>378 ± 9 **</td>
<td>36 ± 3 **</td>
</tr>
<tr>
<td>Ctrl:D+SSTR2a (n=7)</td>
<td>26.4 ± 1.9</td>
<td>368 ± 15</td>
<td>38 ± 3</td>
</tr>
</tbody>
</table>
Table 4-2. Basal plasma counterregulatory hormone levels for rats that underwent hypoglycemia clamp (10 U/kg insulin, 3000 nmol/kg/h SSTR2 antagonist) and control experiments in the absence of insulin (0 U/kg, 3000 nmol/kg/h) at time = -60 min. “N/A” denotes time points at which samples for the particular hormone were not obtained. Data are represented as means ± SEM. *P<0.01 D vs N.

<table>
<thead>
<tr>
<th></th>
<th>glucagon (pg/mL)</th>
<th>corticosterone (ng/mL)</th>
<th>epinephrine (pg/mL)</th>
<th>norepinephrine (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>pilot study</strong> hypoglycemia clamps (10 U/kg insulin, 1500 nmol/kg/h SSTR2 antagonist)**</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>31 ± 9</td>
<td>134 ± 34</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>D</td>
<td>71 ± 14</td>
<td>311 ± 32</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>D+SSTR2a</td>
<td>46 ± 2</td>
<td>309 ± 133</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>hypoglycemia clamps (10 U/kg insulin, 3000 nmol/kg/h SSTR2 antagonist)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>32 ± 4</td>
<td>42 ± 9</td>
<td>55 ± 11</td>
<td>162 ± 26</td>
</tr>
<tr>
<td>D</td>
<td>51 ± 5 *</td>
<td>156 ± 26 *</td>
<td>148 ± 28 *</td>
<td>211 ± 35</td>
</tr>
<tr>
<td>D+SSTR2a</td>
<td>65 ± 5</td>
<td>148 ± 33</td>
<td>134 ± 57</td>
<td>334 ± 60</td>
</tr>
<tr>
<td><strong>hypoglycemia clamps (5 U/kg insulin, 3000 nmol/kg/h SSTR2 antagonist)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>13 ± 6</td>
<td>174 ± 38</td>
<td>40 ± 12</td>
<td>323 ± 50</td>
</tr>
<tr>
<td>D</td>
<td>52 ± 8 *</td>
<td>220 ± 19</td>
<td>93 ± 33</td>
<td>324 ± 29</td>
</tr>
<tr>
<td>D+SSTR2a</td>
<td>37 ± 6</td>
<td>226 ± 45</td>
<td>147 ± 40</td>
<td>282 ± 64</td>
</tr>
<tr>
<td><strong>control experiments (no exogenous insulin administered)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ctrl:N</td>
<td>50 ± 6</td>
<td>165 ± 34</td>
<td>31 ± 15</td>
<td>318 ± 11</td>
</tr>
<tr>
<td>Ctrl:D</td>
<td>45 ± 3</td>
<td>189 ± 54</td>
<td>110 ± 46</td>
<td>310 ± 82</td>
</tr>
<tr>
<td>Ctrl:D+SSTR2a</td>
<td>50 ± 4</td>
<td>245 ± 42</td>
<td>217 ± 65</td>
<td>290 ± 38</td>
</tr>
</tbody>
</table>
Table 4-3. Plasma insulin levels (ng/mL) during hypoglycemia clamps (10 U/kg or 5 U/kg insulin, 3000 nmol/kg/h SSTR2 antagonist). Data are represented as means ± SEM. *P<0.05 D vs N.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>-60</th>
<th>0</th>
<th>60</th>
<th>180</th>
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<tbody>
<tr>
<td><strong>Hypoglycemia clamps using 10 U/kg insulin and 3000 nmol/kg/h SSTR2 antagonist</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>1.0 ± 0.07</td>
<td>0.9 ± 0.07</td>
<td>141.5 ± 8.0</td>
<td>59.6 ± 7.4</td>
</tr>
<tr>
<td>D</td>
<td>0.5 ± 0.04 *</td>
<td>0.5 ± 0.07 *</td>
<td>93.2 ± 7.2 *</td>
<td>24.1 ± 2.9 *</td>
</tr>
<tr>
<td>D+SSTR2a</td>
<td>0.5 ± 0.1</td>
<td>0.7 ± 0.2</td>
<td>107.9 ± 14.1</td>
<td>26.3 ± 4.4</td>
</tr>
</tbody>
</table>

| **Hypoglycemia clamps using 5 U/kg insulin and 3000 nmol/kg/h SSTR2 antagonist** |
| N          | 0.6 ± 0.4  | 0.6 ± 0.2  | 62.4 ± 13.2 | 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  |
Table 4-4. Plasma counterregulatory hormone and insulin levels during control experiments in the absence of insulin (0 U/kg insulin, 3000 nmol/kg/h SSTR2 antagonist). "N/A" denotes time points at which samples for the particular hormone were not obtained. Data are represented as means ± SEM. *P<0.05 D vs N. †P<0.03 D vs D+SSTR2a.

<table>
<thead>
<tr>
<th></th>
<th>Time (min)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>-60</td>
</tr>
<tr>
<td><strong>Plasma glucagon (pg/mL)</strong></td>
<td></td>
</tr>
<tr>
<td>Ctrl:N</td>
<td>50 ± 6</td>
</tr>
<tr>
<td>Ctrl:D</td>
<td>45 ± 3</td>
</tr>
<tr>
<td>Ctrl:D+SSTR2a</td>
<td>50 ± 4</td>
</tr>
<tr>
<td><strong>Plasma corticosterone (ng/mL)</strong></td>
<td></td>
</tr>
<tr>
<td>Ctrl:N</td>
<td>165 ± 31</td>
</tr>
<tr>
<td>Ctrl:D</td>
<td>205 ± 49</td>
</tr>
<tr>
<td>Ctrl:D+SSTR2a</td>
<td>273 ± 46</td>
</tr>
<tr>
<td><strong>Plasma epinephrine (pg/mL)</strong></td>
<td></td>
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<tr>
<td>Ctrl:N</td>
<td>31 ± 15</td>
</tr>
<tr>
<td>Ctrl:D</td>
<td>110 ± 46</td>
</tr>
<tr>
<td>Ctrl:D+SSTR2a</td>
<td>217 ± 65</td>
</tr>
<tr>
<td><strong>Plasma norepinephrine (pg/mL)</strong></td>
<td></td>
</tr>
<tr>
<td>Ctrl:N</td>
<td>356 ± 39</td>
</tr>
<tr>
<td>Ctrl:D</td>
<td>310 ± 82</td>
</tr>
<tr>
<td>Ctrl:D+SSTR2a</td>
<td>290 ± 38</td>
</tr>
<tr>
<td><strong>Plasma insulin (ng/mL)</strong></td>
<td></td>
</tr>
<tr>
<td>Ctrl:N</td>
<td>1.1 ± 0.2*</td>
</tr>
<tr>
<td>Ctrl:D</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>Ctrl:D+SSTR2a</td>
<td>0.5 ± 0.1</td>
</tr>
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Table 4-5. Quantitative mRNA expression of liver PEPCK1 and G6Pase following hypoglycemia clamp (10 U/kg insulin, 3000 nmol/kg/h SSTR2 antagonist) and control experiments in the absence of insulin (0 U/kg, 3000 nmol/kg/h). As per the $2^{-\Delta\Delta Ct}$ method (526), gene expression was normalized to the control group, Ctrl:N. Data are represented as means ± SEM. *P<0.0001 Ctrl:N vs Ctrl:D. †P<0.05 Ctrl:D vs Ctrl:D+SSTR2a. ‡P<0.0001 Ctrl:N vs N. §P<0.01 Ctrl:D+SSTR2a vs D+SSTR2a.

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<th>mRNA expression (fold-change relative to Ctrl:N group)</th>
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<td></td>
<td>PEPCK1</td>
<td>G6Pase</td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ctrl:N</td>
<td>1.000 ± 0.233</td>
<td>1.000 ± 0.301</td>
<td></td>
</tr>
<tr>
<td>Ctrl:D</td>
<td>0.395 ± 0.111</td>
<td>0.160 ± 0.041 *</td>
<td></td>
</tr>
<tr>
<td>Ctrl:D+SSTR2a</td>
<td>1.177 ± 0.280</td>
<td>0.499 ± 0.169 †</td>
<td></td>
</tr>
<tr>
<td>Hypoglycemia</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>N</td>
<td>1.039 ± 0.256</td>
<td>0.171 ± 0.049 ‡</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>1.293 ± 0.444</td>
<td>0.031 ± 0.008</td>
<td></td>
</tr>
<tr>
<td>D+SSTR2a</td>
<td>0.509 ± 0.183</td>
<td>0.033 ± 0.011 §</td>
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</tr>
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</table>
Table 4-6. Metabolic parameters of non-diabetic rats treated with SSTR2 antagonist (N+SSTR2a) that underwent hypoglycemia clamp experiments using the different insulin (U/kg) and SSTR2 antagonist (nmol/kg/h) dosing combinations. Fed blood glucose, body weight, and food intake were measured daily, and the table below shows the mean of values averaged over the last week before the experimental day. Daily blood glucose measurements were obtained using a glucometer. Data are represented as means ± SEM.

<table>
<thead>
<tr>
<th></th>
<th>fed blood glucose (mM)</th>
<th>body weight (g)</th>
<th>food intake (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>N+SSTR2a</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 U/kg, 1500 nmol/kg/h (n=3)</td>
<td>5.7 ± 0.2</td>
<td>419 ± 9</td>
<td>19 ± 1</td>
</tr>
<tr>
<td>10 U/kg, 3000 nmol/kg/h (n=4)</td>
<td>5.2 ± 0.1</td>
<td>405 ± 22</td>
<td>19 ± 1</td>
</tr>
<tr>
<td>5 U/kg, 3000 nmol/kg/h (n=10)</td>
<td>5.3 ± 0.2</td>
<td>396 ± 21</td>
<td>16 ± 1</td>
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</table>
Table 4-7. Pancreatic somatostatin protein levels (pmol/mg protein) and plasma somatostatin levels (pmol/mL) in all hypoglycemic rats. To assess the effects of diabetes and administration of SSTR2 antagonist on somatostatin, available data was combined for each treatment group, irrespective of doses of insulin or SSTR2 antagonist administered. Data are represented as means ± SEM. *P<0.04 D vs N. †P<0.005 D+SSTR2a vs N+SSTR2a. ‡P<0.03 D+SSTR2a vs N+SSTR2a. aP=0.08 D vs N. Additionally, statistical analysis using Two-Way ANOVA shows that there is an effect of diabetes, but not SSTR2 antagonist (P=0.51), on both pancreatic somatostatin (P<0.0004) and plasma somatostatin (P<0.004) values.

<table>
<thead>
<tr>
<th></th>
<th>Pancreatic somatostatin (pmol/mg protein)</th>
<th>Plasma somatostatin (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td></td>
</tr>
<tr>
<td>N</td>
<td>85.3 ± 5.1</td>
<td>11.3 ± 2.4</td>
</tr>
<tr>
<td>D</td>
<td>121.1 ± 12.9 *</td>
<td>18.4 ± 2.3 a</td>
</tr>
<tr>
<td>N+SSTR2a</td>
<td>78.9 ± 7.8</td>
<td>12.0 ± 2.7</td>
</tr>
<tr>
<td>D+SSTR2a</td>
<td>130.4 ± 10.6 †</td>
<td>21.2 ± 2.2 ‡</td>
</tr>
</tbody>
</table>
Chapter 5

Study 2: Amelioration of hypoglycemia using SSTR2 antagonism

5 Study 2

5.1 Aims

Based on our prior observation that SSTR2 antagonism restored glucagon and corticosterone counterregulation in acutely hypoglycemic diabetic rats, we wished to test whether SSTR2 antagonism could ameliorate subsequent hypoglycemia by restoring blood glucose levels to euglycemia, and whether this glycemic rescue could be attributed to improved hormone counterregulation, despite exposure to recurrent hypoglycemia. Thus the aim of the present study was to determine whether counterregulatory hormone responses can be restored by SSTR2 antagonism in diabetic rats exposed to recurrent hypoglycemia and if such improvements can lessen the severity of subsequent hypoglycemia. Recurrent hypoglycemia is associated with marked impairments of counterregulatory hormone responses. Glucagon counterregulation in diabetes is often absent (165;166), thus it has not been shown to be further worsened by recurrent hypoglycemia (203;227;264). However, there is evidence demonstrating markedly diminished epinephrine counterregulation following recurrent hypoglycemia in diabetic subjects (203;205;220;227;261;263;264). Cortisol responses following recurrent hypoglycemia are less consistent, having been found unaltered (203;264) or decreased (205;227) in diabetes. In the present study, diabetic rats were subjected to 5 prior episodes of hypoglycemia (i.e. recurrent hypoglycemia treatment) in an attempt to create a
model of markedly defective counterregulation. Our present results demonstrate that
SSTR2 antagonism promotes the recovery of hypoglycemia in recurrently hypoglycemic
diabetic rats and that improved glucagon counterregulation may be responsible, at least in
part, for this amelioration.

5.2 Experimental methods

5.2.1 Study design

Figure 5-1 illustrates the study design for the main protocol. As will be described in detail
below, two groups of STZ-diabetic rats were used in this study. Initial pilot studies
examined the effect of only 2 consecutive days of hypoglycemia (i.e. without prior recurrent
hypoglycemia treatment) and is presented in Figure 5-2 and Figure 5-3.

5.2.2 Recurrent hypoglycemia treatment

Rats were subjected to 5 episodes of recurrent hypoglycemia over 3 consecutive days (i.e. 2
episodes per day on the first two days and 1 episode on the third day). Rats were partially
fasted overnight prior to each day of recurrent hypoglycemia treatment. Partial fasting
consisted of 10-15 g of rat chow (i.e. approximately 25-40% of ad libitum consumption)
and free access to 5% sucrose water. Basal blood glucose was measured at the 0 min time
point, and regular insulin (20 U/kg; Humulin R, Eli Lilly, Indianapolis, IN) was injected
subcutaneously to induce each episode of 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 from hypoglycemia. One blood glucose sample was obtained during the 
break to monitor glycemia. At 240 min, rats were given a second insulin injection as above 
and remained at hypoglycemic until 420 min. Food and sucrose water were fed to aid 
recovery after hypoglycemia treatment.

5.2.3 Partial overnight fasting prior to hypoglycemia experiments
Rats were partially fasted overnight prior to each experimental day. This food restriction 
consisted of 10-15 g of rat chow (i.e. approximately 25-40% of ad libitum consumption) 
given overnight when rats are awake. Fasting rats in this manner was necessary for three 
purposes: (i) it allowed target hypoglycemia to be achieved more easily during recurrent 
hypoglycemia treatment as compared to non-fasted conditions; (ii) it allowed rats to 
preserve some glycogen stores to facilitate recovery from hypoglycemia during 
Experimental Days 1 and 2; (iii) and it allowed for better control of body weight and initial 
glycemia, which was important since insulin dosing was dependent on these variables and 
we wished to keep the amount of insulin administered consistent between the 2 
experimental days. Limitations to this fasting method included an inability to precisely 
determine the time at which rats ate their rationed chow; we narrowed the window of 
rationed chow availability to between 22:00 (when chow was weighed and rationed) and 
09:00 (when rat cages where changed, any uneaten food removed, and 5% sucrose water 
replaced with regular water).

5.2.4 Hypoglycemia on Experimental Days 1 and 2
Following recurrent hypoglycemia treatment, each rat subsequently underwent 2 days of 
experimentation. The protocol for experimental day 1 (ExptD1) and experimental day 2 
(Expt2) is summarized in Figure 5-1. On the morning of the experiments, rats were 
weighed and had their cages changed, connected to infusion lines, and acclimatized for a
minimum of 2 h prior to the start of experimentation. Basal blood samples for glucose and hormones were taken at the start of the experiment (i.e. \( t = -60 \) min) from freely moving, conscious rats. Extreme care was taken to avoid stresses to the rats during the experiments since it is well known that even minimal disturbances caused by sounds, scents, and vibrations can increase the release of the stress hormones measured. On ExptD1, 0.9% saline infusion (1 mL/h) was started after basal samples were obtained at the -60 min time point. Blood glucose was measured using a glucose analyzer in duplicate at times -60, -40, -20, 0, and every 10 min thereafter until 240 min. Blood samples were obtained from the carotid catheter at regular time intervals throughout the experiment for measurement of plasma hormone levels. 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, and a primed intravenous insulin infusion (50 mU/kg/min) was commenced and terminated specifically for each rat at the experimenter’s discretion when target hypoglycemia (<4.0 mM) was approached. Infusions were delivered via digital pumps (Harvard Apparatus PHD 22/2000 syringe pumps, Holliston, MA), and both the volume of insulin infused and the time at which the infusion was stopped were recorded. The purpose of Experimental Day 1 was to precisely determine the minimal amount of insulin necessary to induce hypoglycemia in a particular rat. Determining the insulin dosage specifically for each rat was necessary since the insulin sensitivities of these diabetic rats varied. No glucose infusions or SSTR2 antagonist were given on the experimental days.

On ExptD2, rats were divided into the SSTR2 antagonist treatment group (saline-SSTR2a, \( n = 13 \)) or control group (saline-saline, \( n = 7 \)). In each rat, the same insulin administration
regime was used as ExptD1. It is important to emphasize that: (i) the same rat was used in both ExptD1 and ExptD2 for comparisons; (ii) the SSTR2 antagonist treatment group and the control group represent two different groups of rats. In the treatment group, SSTR2 antagonist infusion (3000 nmol/kg/min at 1 mL/h) was commenced at t=-60 min and continued for 5-h duration of the experiment to determine the effect of SSTR2 antagonism on the extent (i.e. depth and duration) of hypoglycemia. This dose of SSTR2 antagonist was chosen because it normalized glucose and corticosterone responses to acute hypoglycemia in diabetic rats and did not elicit hyperglycemia or marked elevations in counterregulatory during an absence of hypoglycemia (Study 1). In the control group, saline was infused without SSTR2 antagonist to control for the repeated bout of hypoglycemia (i.e. saline infusions on both ExptD1 and ExptD2). Body weight, initial glycemia, and overnight food intake were controlled for both days in all rats. At the end of 240 min on ExptD2, rats were quickly euthanized by decapitation. Rats that required glucose infusion due to severe hypoglycemia (i.e. < 1.6 mM) were excluded from the data presented.

5.3 Results

5.3.1 Pilot Studies: How the present method was established

Initial pilot studies examined the effect of only 2 consecutive days of hypoglycemia (i.e. without prior recurrent hypoglycemia treatment) in STZ-diabetic rats in which insulin was administered subcutaneously with repeated staggered injections (Figure 5-2; n=4; average of 4.8 U insulin injected) or intravenously via bolus and infusion (Figure 5-3; n=3; average of 4.9 U insulin administered). Repeated, staggered subcutaneous injections proved to be problematic since: i) unwanted stress was caused, and ii) precise dosing was difficult to
administer. In both studies, as in the data presented below, a minimal amount of insulin to
induce hypoglycemia was administered on the first day, the same amount of insulin was
given on the second day to the same rat, and no glucose infusion was given so that
recovery could be assessed. In these pilot studies, rats were overnight fasted. In both
studies, one prior bout of hypoglycemia the day before greatly worsened hypoglycemia
induced with the same amount of insulin on the next consecutive day as was apparent both
by the measurement of blood glucose levels (Figure 5-2A, Figure 5-3A) as well as the
observable behavioural condition of the rats (i.e. immobilization, and in many rats, severe
twitches, which may have been seizures). One antecedent bout of hypoglycemia was
sufficient to markedly diminish glucagon responses to subsequent hypoglycemia (Figure
5-2B, Figure 5-3B). We reasoned that a protocol that involved recurrent hypoglycemia
treatment in a clamped setting prior to experimentation would be useful for the following
reasons: i) from a practical standpoint, several prior repeated episodes of hypoglycemia
would help to minimize the glycemic variation between subsequent bouts of hypoglycemia
(i.e. we hypothesized a habituation effect); ii) it would allow us to evaluate the effect of
SSTR2 antagonist in a established model of severe counterregulatory impairment; and iii) as
we hypothesized that epinephrine counterregulation would be diminished in recurrently
hypoglycemic diabetic rats (unlike in our acute hypoglycemia setting in Study 1), it would
allow us to evaluate the effect of SSTR2 antagonist on epinephrine counterregulation. Due
to the marked effect of one antecedent bout of hypoglycemia on impairing hypoglycemic
recovery, we also decided to partially fast, instead of fully fast, rats overnight.

5.3.2 Daily blood glucose, body weight, and food intake
Metabolic parameters such as fed morning blood glucose, body weight, and food intake
were measured daily to monitor the health of the rats and to ensure that diabetic rats were
of a similar diabetic state leading up to recurrent hypoglycemia treatment. In the week prior to recurrent hypoglycemia, fed morning hyperglycemia, body weight, and daily food intake were the same in rats that would later be divided into SSTR2 antagonist treatment and controls groups for hypoglycemia experiments (Table 5-1).

5.3.3 **Glycemia during recurrent hypoglycemia treatment**
Both groups achieved 5 similar episodes of recurrent hypoglycemia over 3 days (3.0±0.5 mM for an average of 90 min per episode; Figure 5-4).

5.3.4 **Basal blood glucose and plasma hormone levels after recurrent hypoglycemia treatment**
On the morning of Experimental Days 1 and 2, body weight and initial glycemia did not differ between rats (Table 5-2). Thus, all rats had similar metabolic starting points following recurrent hypoglycemia, which allowed for baseline control conditions. Basal plasma glucagon, epinephrine, norepinephrine, and insulin were also similar in all rats (Table 5-3). Oddly, basal corticosterone of ExptD2-SSTR2a was lower compared with its respective ExptD1 \( (P<0.03) \), whereas basal corticosterone was unchanged in controls (Table 5-3). This is unusual because this change cannot be attributed to any changes in the metabolic parameters measured, to the levels of basal hormones measured, to recurrent hypoglycemia, or to SSTR2 antagonist since this was before the antagonist was administered.

5.3.5 **Blood glucose and plasma insulin levels during Experimental Days 1 and 2**
On ExptD1, insulin induced a similar rate of decline in blood glucose levels to <4.0 mM by 90 min in both treatment (Figure 5-5) and control (Figure 5-6) groups. The hypoglycemic nadirs were likewise the same in both treatment (Figure 5-7A) and control groups (Figure 5-7B) on ExptD1. The control group had a longer duration of hypoglycemia compared to
the treatment group. When SSTR2 antagonist was administered on ExptD2 with the same insulin administration regime (i.e. same bolus dose and same infusion dose, rate, and timing of administration) as ExptD1, a similar glycemic decline from hyperglycemia ensued, but the depth and duration of hypoglycemia was significantly less when SSTR2 antagonist was given (Figure 5-7A). This demonstrates that SSTR2 antagonism does not affect insulin sensitivity or its glycemia lowering effect. SSTR2a rats recovered from hypoglycemia more quickly, and the hypoglycemic nadir was less severe in ExptD2-SSTR2a (3.7±0.3 mM) than on the preceding day (2.9±0.1 mM; P<0.01). The extent of hypoglycemia, calculated as the area under the curve below 4.0 mM, was significantly reduced in the SSTR2 antagonist treatment group (P<0.001; Figure 5-7A). Analysis of time points after glycemia in both groups fell below 7.0 mM (i.e. t=50-240min) or 4.0 mM (i.e. t=110-240 min) with Repeated Measures ANOVA revealed a significant effect of SSTR2 antagonist (P<0.02 and P<0.005, respectively). Without the SSTR2 antagonist, there was no improvement in hypoglycemic recovery (Figure 5-7B). Controls had similar hypoglycemia on both days (nadirs: 2.8±0.4 mM).

As we carefully endeavoured, similar amounts of insulin (via bolus, infusion, and total amount given) were administered to both groups on both days (Table 5-2). Giving the same amount of insulin on both experimental days within a group was important so that any changes observed with glycemia would not be attributed to the amount of insulin administered. This resulted in similar plasma insulin profiles for both groups (Table 5-4).

5.3.6 Counterregulatory hormones levels during Experimental Days 1 and 2

Following recurrent hypoglycemia, glucagon responses on Experimental Day 1 in both groups (Figure 5-8) were modest. Figure 5-8A shows all rats that received SSTR2 antagonist on ExptD2 and their respective glucagon responses on ExptD1. Figure 5-8B
omits six rats that did not experience hypoglycemia as a result of their SSTR2 antagonist treatment on ExptD2 and their respective glucagon responses on ExptD1 (i.e. Figure 5-8B shows data for both experimental days of rats whose blood glucose levels dipped below the normal physiological threshold of 4.0 mM in response to insulin administration despite SSTR2 antagonist treatment on ExptD2). Figure 5-8C shows the glucagon responses of control rats (i.e. no SSTR2 antagonist administered on ExptD2). It should be noted that none of the ExptD2-control rats had hypoglycemia prevented. SSTR2 antagonist significantly increased plasma glucagon on ExptD2 by 3-fold as compared to ExptD1 ($P<0.04$, Figure 5-8A) and had a strong tendency to improve plasma glucagon by 2.2-fold in rats that still experienced hypoglycemia ($P=0.05$, Figure 5-8B), whereas the glucagon response in controls remained markedly attenuated and exhibited a trend to be decreased, although this was not statistically significant (Figure 5-8C).

Epinephrine responses were significantly lower in ExptD2-SSTR2a rats than on their respective ExptD1 (Figure 5-9A). We attribute this attenuated epinephrine response to the improvement in hypoglycemic recovery on ExptD2 due to SSTR2 antagonism; since many of these rats did not experience marked hypoglycemia, there was no need for them to respond with robust epinephrine counterregulation. When epinephrine responses were analyzed having omitted the six rats in which hypoglycemia was prevented on ExptD2 due to administration of SSTR2 antagonist, it is observed that SSTR2 antagonist preserves epinephrine counterregulation to recurrent hypoglycemia (Figure 5-9B). In contrast, ExptD2-controls had a significantly blunted epinephrine counterregulation ($38\%$ reduction) compared to their respective responses on ExptD1 ($P<0.02$; Figure 5-9C). As mentioned above, none of the ExptD2-control rats had hypoglycemia prevented. This suggests that
SSTR2 antagonism can help preserve the epinephrine response in recurrently hypoglycemic rats when exposed to subsequent hypoglycemia.

Corticosterone responses likewise were decreased in ExptD2-controls by 46% compared to their responses on ExptD1 ($P<0.04$, Figure 5-10C). However, treatment in ExptD2-SSTR2a preserved the corticosterone response to hypoglycemia following recurrent hypoglycemia (Figure 5-10A). Omission of the six rats in which hypoglycemia was prevented did not significantly alter the corticosterone profile of the ExptD2-SSTR2a group, although these rats demonstrated a strong tendency for increased corticosterone counterregulation ($P=0.05$; Figure 5-10B).

Despite similar metabolic profiles (i.e. body weight, severity of diabetic hyperglycemia at baseline), similar recurrent hypoglycemia treatment, and similar insulin administration and plasma levels, ExptD1 epinephrine and corticosterone responses to hypoglycemia were greater in controls than in the treatment group. Similar counterregulatory hormone responses following recurrent hypoglycemia were expected on ExptD1, which occurred before SSTR2 antagonist treatment, since rats had similar metabolic profiles and were treated in the same way up until this point. We attribute the larger epinephrine and corticosterone responses of the ExptD1 control group to a greater extent of hypoglycemia (Figure 5-7B) as compared with ExptD1 in the treatment group (Figure 5-7A). This supports the idea that different rats have different insulin sensitivities; given the same amount of insulin, some rats may be more susceptible to hypoglycemia than others. Thus, it was important that we used the method of comparing the same rat to the effects of recurrent hypoglycemia on ExptD1 and ExptD2, with (i.e. treatment group) and without (i.e. control group) SSTR2 antagonist.
Norepinephrine levels were similar on both experimental days in each of the groups (Figure 5-11).

Figure 5-12 compares the peak incremental hormone responses to acute hypoglycemia from Study 1 and recurrent hypoglycemia from Study 2 in diabetic control rats and diabetic rats treated with SSTR2 antagonist and will be discussed below.

5.4 Discussion

5.4.1 Amelioration of hypoglycemia with SSTR2 antagonism

We demonstrate that SSTR2 antagonism promotes the recovery of hypoglycemia by reducing both the depth and duration of hypoglycemia in recurrently hypoglycemic diabetic rats. Whereas control animals subjected to recurrent hypoglycemia had glycemic nadirs of 2.8±0.4 mM on both experimental days, rats given SSTR2 antagonist had a significantly improved glycemic profile as demonstrated by a shorter duration of hypoglycemia and an elevated glycemic nadir of 3.7±0.3 mM. Lessening the hypoglycemic nadir is important to preserve brain function. An early study demonstrated that hypoglycemia at 3.3 mM reduced cognitive function as compared with euglycemia in diabetic subjects (620). Blood-to-brain glucose transport is a direct function of the arterial plasma glucose concentration, and during hypoglycemia blood-to-brain glucose transport becomes limiting to brain glucose metabolism and, therefore, function (4). The American Diabetes Association Workgroup on Hypoglycemia has emphasized not only the inherent danger of the nadir glucose concentration and the duration of hypoglycemia but also of the frequency of hypoglycemic events (1). Some (151;621;622) have challenged the substantiality of defining hypoglycemia as ≤3.9 mM, as published by the American Diabetes Association Workgroup.
on Hypoglycemia (1). Amiel et al. suggest that glycemic excursions in the range of 3.5–4.0 mM, provided that further glycemic decline is prevented, is of little clinical significance (151). Swinnen et al. report that defining hypoglycemia at a higher glucose level of 3.9 mM overestimates the prevalence of asymptomatic episodes of hypoglycemia and suggest a lower glycemic cut-off (622). Frier suggests that a cut-off level of 3.5 mM or lower to define the onset of hypoglycemia would be clinically appropriate since cognitive function is not impaired, and symptoms are often not generated, until glycemia approaches 3.2 mM (621). If this were indeed the case, then our present data would illustrate that SSTR2 antagonist treatment could not only facilitate recovery from low blood glucose levels but also prevent the onset of hypoglycemia. However, it should be noted that a more conservative definition of hypoglycemia (i.e. at the biochemical level of ≤3.9 mM) has its merits as rebutted by Cryer (623). Hypoglycemia, even when unaccompanied by symptoms, leads to defective glucose counterregulation and eventually hypoglycemia unawareness (63;168). Since these episodes substantially increase the risk of subsequent hypoglycemia, all hypoglycemic events can harm the individual with diabetes in the short or long term (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 (225). Thus, developing a means to promote hypoglycemic recovery is of vital importance.

Hypoglycemia unawareness is the development of neuroglycopenia without appropriate prior autonomic warning symptoms (232;624) and contributes to the increased risk of recurrent hypoglycemia (63). In type 1 diabetic patients, avoidance of hypoglycemia could restore hypoglycemia awareness. Improved symptom scores, increased glycemic thresholds for responses, and/or improved epinephrine counterregulation were observed after 2 weeks
However, the restoration of awareness would often come at the cost of less strict glycemic control as measured by increased hemoglobin A$_{1c}$ levels (241;625;626). Thus, our findings suggest a means by which hypoglycemia can be ameliorated without compromising glycemic management.

So how might SSTR2 antagonism facilitate the recovery of hypoglycemia? We demonstrate that facilitated recovery from hypoglycemia using SSTR2 antagonist is associated with an improvements in glucagon, epinephrine, and corticosterone responses to recurrent hypoglycemia. As will be discussed in section 5.4.2, improvements of hormone counterregulation could increase glucose production during hypoglycemia to help restore euglycemia, thus ameliorating hypoglycemia via SSTR2 antagonism.

It has been demonstrated that central administration of somatostatin has no effect on plasma glucose levels under basal conditions. However, when somatostatin was administered into the central nervous system in conjunction with β-endorphin or bombesin, the hyperglycemia that would typically ensue from each of these substances was abolished (627;628). An elevation of blood glucose levels is achieved by increasing glucose production and decreasing glucose clearance. From Study 1, 4-h infusion of SSTR2 antagonist in diabetic rats during hypoglycemia yielded no changes in growth hormone levels as compared with saline infused controls, thus it is unlikely that growth hormone-induced hepatic glucose output contributed to improved hypoglycemic recovery. The inability of somatostatin to directly affect glucose turnover both in vivo in dogs (629) and in vitro in rat hepatocytes (616) has been reported by much earlier studies by Cherrington et al. In dogs, infusion of somatostatin with intraportal replacement co-infusions insulin and glucagon did not affect glucose production, which suggests that somatostatin may affect glucose production indirectly via its effects on insulin and glucagon, but not directly (629).
However, the lack of effect of somatostatin reported in these studies was during the basal, non-stimulated state and is does not necessarily represent conditions of hepatic stimulation. In perfused rat liver, somatostatin inhibited glucagon-stimulated, but not basal, hepatic glucose output (630). Since somatostatin also blunted cAMP-induced glucose output, the authors suggest the effect of somatostatin to suppress stimulated hepatic glucose release is mediated via cAMP (630), which is consistent with a known mechanism of action of somatostatin. Furthermore, in isolated hepatocytes, somatostatin inhibited both glucagon-stimulated glycogenolysis and gluconeogenesis by 40-50%, but somatostatin had no effect on epinephrine-stimulated glycogenolysis (631). At present, however, there is no known direct effect of SSTR2 antagonism on hepatic glucose production. SSTR1 (271) and SSTR3 (373), but not SSTR2 (418;632), have been detected on hepatocytes, and the SSTR2 antagonist has no reactivity with SSTR1 and 10-fold less binding affinity with SSTR3 (509). We demonstrate that SSTR2 antagonism did not affect the mRNA expression of gluconeogenic enzyme PEPCK in diabetic rats during basal conditions or in response to hypoglycemia (Study 1). Similarly, SSTR2 antagonist did not affect expression of G6Pase following hypoglycemia but increased G6Pase expression during basal conditions (Study 1). In another study, however, SSTR2-deficient mice were shown to have increased mRNA expression of PEPCK, but not G6Pase (592).

It has been postulated that an increase in glucose clearance may be attributed to some extrapancreatic effect of somatostatin (633). In one study, somatostatin infusion did not increase whole-body glucose disposal in non-diabetic humans during a hyperinsulinemic-euglycemic clamp (298). In contrast, a direct effect of somatostatin to enhance insulin-stimulated muscle glucose uptake, but not basal muscle glucose uptake, was demonstrated in non-diabetic humans (634). During a hyperinsulinemic-euglycemic clamp, local forearm
perfusion (via the brachial artery) with somatostatin elicited a significant 55% increase in
insulin-stimulated forearm glucose uptake as compared with systemic venous somatostatin
infusion without affecting the exogenous glucose requirement or plasma insulin, glucagon,
or growth hormone levels. The authors concluded that somatostatin may increase insulin-
stimulated glucose uptake in muscle via local mechanisms (634). Thus, it is plausible that
inhibition of somatostatin in the present study could contribute to reduced muscle glucose
uptake during hypoglycemia, thus increasing blood glucose levels. Evidence of SSTR
subtypes on skeletal muscle is scarce, but one group has reported detection of SSTR2,
SSTR3, and SSTR4 mRNA in rat skeletal muscle (635) while another group has evidence of
SSTR5 mRNA in skeletal muscle (636).

5.4.2 SSTR2 antagonism improves counterregulation following recurrent hypoglycemia
Recurrent hypoglycemia is well known to impair counterregulatory hormone responses in
both non-diabetic (227;265;637-640) and diabetic (203;205;220;227;261;263;264)
subjects. We also demonstrate that the peak incremental glucagon ($P<0.005$) and
epinephrine ($P<0.001$) responses to subsequent hypoglycemia are blunted in recurrently
hypoglycemic rats as compared to rats subject to acute hypoglycemia (Figure 5-12).

5.4.2.1 SSTR2 antagonism increases glucagon counterregulation.
Presently, we provide evidence that SSTR2 antagonism increases plasma glucagon levels in
recurrently hypoglycemic diabetic rats. In contrast, the glucagon response to recurrent
hypoglycemia was absent in control rats without SSTR2 antagonist. In the present study,
we were unable to collect and analyze pancreata from our experimental animals. However,
we previously observed increased pancreatic prosomatostatin mRNA expression in diabetic
rats as compared to non-diabetic rats (205). After 7 episodes of recurrent hypoglycemia in
diabetic rats, the expression of somatostatin was slightly but not significantly decreased.
compared to diabetic controls without recurrent hypoglycemia but remained partially elevated in as compared with non-diabetic controls, which indicates partial normalization of pancreatic somatostatin expression following recurrent hypoglycemia (205). In contrast, 7 recurrent episodes of recurrent hyperinsulinemia, *per se*, decreased but did not fully normalize pancreatic somatostatin mRNA, which demonstrates that repeated insulin administration, and not repeated hypoglycemia, may help to partially normalize pancreatic somatostatin expression. In these diabetic rats that received recurrent hyperinsulinemic treatment, with or without recurrent hypoglycemia, and had partially normalized pancreatic somatostatin expression, glucagon responses to subsequent hypoglycemia was improved as compared with acutely hypoglycemic diabetic controls but still impaired compared to non-diabetic controls (205). Thus, as with acute hypoglycemia, increased pancreatic somatostatin may play a role in impairing glucagon release in response to repeated hypoglycemia.

It should be noted that although SSTR2 antagonism improved glucagon responsiveness in recurrently hypoglycemic rats by 3-fold, this peak incremental response was much less than the glucagon response in SSTR2 antagonist-treated diabetic rats during acute hypoglycemia from Study 1 (30±10 vs. 228±57 pg/mL; Figure 5-12A). In a separate study we previously demonstrated that chronic, modest insulin treatment in STZ-diabetic rats does not normalize glucagon counterregulation following recurrent hypoglycemia (641). Thus, recurrent hypoglycemia still overwhelmingly attenuates glucagon counterregulation, irrespective of insulin treatment, and we suggest that SSTR2 antagonism may offer significant improvement.
5.4.2.2  **SSTR2 antagonism preserves epinephrine counterregulation.**

A very important defect of counterregulation in many diabetic individuals is the poor response of epinephrine, especially in conjunction with an absent glucagon response and hypoglycemia unawareness (3). Previously, we observed a deficiency of the epinephrine response to recurrent hypoglycemia in STZ-diabetic rats (205), and this defect was attributed, in part, to deficiencies in the mRNA expression of two key catecholamine-synthesizing enzymes (220). Furthermore, it has also been shown that although BB diabetic rats had normal total catecholamine content in the adrenal medulla, they showed a hypofunctional adrenomedullary chromaffin cell release of epinephrine (642). A central effect of somatostatin on regulating epinephrine cannot be ruled out. Prior studies have demonstrated that analogues of somatostatin inhibit the plasma catecholamine response to central glucopenia and to central administration of carbachol and bombesin (528;643).

We presently hypothesized that antagonizing somatostatin may play a role in increasing secretion of adrenomedullary epinephrine in response to recurrent hypoglycemia. In our prior acute hypoglycemia study (Study 1), we observed no effect of the SSTR2 antagonist on epinephrine levels in diabetic rats, which had the same epinephrine response as non-diabetic controls. We postulated that no further enhancement by SSTR2 antagonist could be observed in those rats because the maximal physiological epinephrine response was already achieved. As compared to our acute hypoglycemia study, peak increments in plasma epinephrine levels following recurrent hypoglycemia were markedly reduced (acute diabetic control: 3845±358 vs. recurrently hypoglycemic diabetic control: 1013±270 pg/mL), demonstrating that our recurrent hypoglycemia treatment generated a model of defective counterregulation. The findings from the present study suggest that: (i) recurrent hypoglycemia further decreased epinephrine levels in controls; (ii) when hypoglycemia is
prevented, administration of SSTR2 antagonist does not increase plasma epinephrine levels; but that (iii) SSTR2 antagonism helps to preserve the epinephrine response from further decrement in rats whose glycemia dips below the normal physiological range, which may aid in the promoted recovery from hypoglycemia.

SSTR2 has been widely identified in the adrenal medulla (390;397-399). It has been shown that somatostatin inhibits acetylcholine-stimulated release of epinephrine from the adrenal medulla (644;645). This is the mechanism whereby epinephrine is released during hypoglycemia and other stressors (646). Somatostatin is postulated to inhibit receptor-coupled signalling initiated by acetylcholine-nicotinic receptor binding, subsequent membrane depolarization, and intracellular calcium increase (647-649), resulting in the consequent inhibition of adrenomedullary epinephrine secretion from the adrenal medulla. Furthermore it has been shown that although diabetic rats had normal catecholamine content in the adrenal medulla, a defect in the secretory mechanism of epinephrine from adrenomedullary chromaffin cells was observed (642). Therefore, we hypothesize that administration of the SSTR2 antagonist may improve or normalize the epinephrine response to hypoglycemia in diabetic rats. Co-storage and co-secretion of somatostatin with catecholamines in chromaffin cells of the adrenal medulla granules have been demonstrated (650). Thus, somatostatin may have the physiologic role of a negative feedback mechanism for catecholamine release in the adrenal medulla (647). In addition, it is well known that somatostatin administered directly in the brain reduces stimulated epinephrine responses. Intracerebroventricular administration of somatostatin or its analogs reduces stress-induced plasma epinephrine levels in non-diabetic rats and non-diabetic and diabetic dogs (169;528;643;651;652). Thus, in addition to the direct effect of SSTR2 blockade at the
adrenal medulla, it is also possible that inhibition of SSTR2 in the brain may indirectly increase plasma epinephrine responses to hypoglycemia.

An improvement in the epinephrine response might also contribute to the amelioration of hypoglycemia as observed in this study. It has been reported that in many type 2 diabetic individuals with insulin resistance, the lipolytic effects of epinephrine outweigh the effects of insulin on adipose tissue (186). Plasma free fatty acids increase in response to hypoglycemia in type 2 diabetes (167;183;186) but not in type 1 diabetes (653). Epinephrine secretion during hypoglycemia may therefore have a greater protective effect in insulin-resistant subjects by promoting metabolic substrate release rather than storage. Epinephrine also stimulates release of glucose from the kidney, and, in subjects who have a deficient glucagon response to hypoglycemia, this has been suggested to compensate for their impaired hepatic glucose output (213).

Norepinephrine counterregulation was not affected by recurrent hypoglycemia; peak incremental norepinephrine responses were similar in diabetic control rats following acute and recurrent hypoglycemia (565±43 vs. 604±129 pg/mL, respectively). SSTR2 antagonism had no effect on plasma norepinephrine levels in recurrently hypoglycemic rats. This finding is consistent with the report that intracerebroventricular administration of somatostatin or its analogs does not reduce stress-induced plasma elevations of plasma norepinephrine (654).

5.4.2.3 SSTR2 antagonism preserves corticosterone counterregulation.

The glucocorticoid response to hypoglycemia plays a role in reinstating euglycemia, is important during prolonged hypoglycemia, but may not play a critical role in rapid counterregulation if other responses are intact (3). We have demonstrated that SSTR2
antagonism can improve the corticosterone response to acute hypoglycemia in diabetic rats (Study 1). In this study, we report that SSTR2 antagonist preserves the corticosterone response to recurrent hypoglycemia whereas rats exposed to recurrent hypoglycemia without SSTR2 antagonism display attenuated corticosterone counterregulation. Previously, we demonstrated that insulin-treated STZ-diabetic rats had an absent corticosterone response following recurrent hypoglycemia (641). We attributed this defect mainly to a lack of suppression of hippocampal mineralocorticoid receptor mRNA expression and a consequent lack of increase of hypothalamic CRH mRNA expression in response to hypoglycemia (655). Intracerebroventricular administration of somatostatin blunts stress-induced plasma cortisol levels in non-diabetic and diabetic dogs (169;528). Furthermore, central administration of somatostatin inhibits pituitary release of ACTH (656), which stimulates adrenocortical release of corticosterone. SSTR2 have been localized in several brain regions and specifically in areas pertaining to HPA activity – the hypothalamic paraventricular nucleus (420) and ACTH cells in the anterior pituitary (561). Interestingly, somatostatin inhibits CRH and ACTH release (271;294;657;658), which regulate corticosterone secretion. At present, the precise mechanism of somatostatin on its regulation of the HPA axis in unknown, but we postulate that somatostatin may play a role in regulating the stress response to hypoglycemia.

5.4.3 Discussion of other mechanisms for reduced counterregulation following recurrent hypoglycemia

Hypoglycemia diminishes hormone, symptom, and cognitive responses to subsequent hypoglycemia in type 1 diabetic (203;223;226), type 2 diabetic (168), and non-diabetic individuals. Recurrent hypoglycemia alters the thresholds for these responses such that lower blood glucose levels must be reached before counterregulation is initiated (4). Notably, the epinephrine response to hypoglycemia is diminished by recurrent
hypoglycemia, which plays a role both in defective glucose counterregulation due to absent glucagon counterregulation and hypoglycemia unawareness. Taken together, recurrent hypoglycemia and the associated impairment of epinephrine counterregulation substantially contribute to hypoglycemia-associated autonomic failure (HAAF) (63). Several different mechanisms have been hypothesized to contribute to these impairments associated with recurrent hypoglycemia.

Several studies have demonstrated that recurrent hypoglycemia alters glucose sensing in the brain (193). The VMH contains glucose-sensing neurons (24;40;659) and also is a critical region for the initiation of hypoglycemic counterregulation (34-36). Glucose-inhibited (GI) neurons in the VMH increase their firing rate when extracellular glucose levels decrease (40). Recurrent hypoglycemia impairs the response of VMH GI neurons to low glucose conditions and lowers the glucose threshold at which neurons are activated (263;660). Chan et al. have demonstrated that increased VMH GABAergic tone suppresses counterregulation following recurrent hypoglycemia (661). This effect of increased GABA inhibitory tone resulting from recurrent hypoglycemia is believed to be mediated via glucose-excitatory (GE) neurons in the VMH. A recent hypothesis by McCrimmon posits that recurrent hypoglycemia alters the balance between the 2 subpopulations of glucose sensing neurons: the GI neurons and GE neurons. It is hypothesized that GI neurons, which act to amplify counterregulation, are less likely to be active and/or GE neurons, which act to suppress counterregulation, are more likely to be active following recurrent hypoglycemia (193). This hypothesis is consistent with the studies mentioned above which demonstrate that in the VMH, recurrent hypoglycemia increased GABAergic inhibitory tone (which is postulated to be GE neuron-mediated) and decreased AMPK activity (which is postulated to be GI neuron-mediated) (193). Thus, the decreased counterregulatory hormone responses
observed in the recurrently hypoglycemic rats of the present study may be due to recurrent-hypoglycemia induced alterations in brain glucose sensing.

In addition to the VMH, other brain areas have also been implicated in regulating counterregulation to hypoglycemia. Integration of behavioural responses to hypoglycemia is associated with brain networks that include the amygdala, brain stem, orbitofrontal cortex (cognitive decision-making), and ventral striatum (reward centre) (662). Hypoglycemia unaware subjects, who also had decreased epinephrine counterregulation, demonstrated reduced responses in these networks, and it is hypothesized that habituation of these behavioural responses may contribute to hypoglycemia unawareness (662). Furthermore, the dorsal midline thalamus has been demonstrated to play an inhibitory role following recurrent hypoglycemia and contribute to HAAF by reducing both symptom and epinephrine responses to subsequent hypoglycemia (663). Thus, the counterregulatory defects observed following recurrent hypoglycemia are regulated by complex neural integration of both glucose sensing and afferent neural pathways that initiate counterregulatory responses.

Another hypothesized mechanism relates to increased transport of glucose or other fuels to the brain following recurrent hypoglycemia. When the brain fails to sense a local decrease in glucose concentrations as a result of increased blood-to-brain transport of glucose or alternate fuels, the brain reduces its initiation of counterregulatory hormone responses (664;665). Boyle et al. have demonstrated adaptations that maintain brain glucose uptake at normal levels following recurrent hypoglycemia and in type 1 diabetic individuals, thereby reducing neuroendocrine counterregulatory responses (178;666). Prolonged, chronic hypoglycemia of at least 3 days increases glucose transporter (GLUT)-1 mRNA and protein at the blood-brain-barrier and glucose uptake in rats (177;667;668). However, this
hypothesis has been challenged in light of evidence that demonstrated that 24 h of hypoglycemia in humans blunted counterregulation to subsequent hypoglycemia but did not affect glucose transport, glucose metabolism, or blood flow in the brain (669). Furthermore, recurrent hypoglycemia of a shorter duration blunts hormone counterregulation, but increases of local glucose concentrations in various brain regions during subsequent hypoglycemia have not been observed. Thus, recurrent intermittent hypoglycemia may not be a potent enough stimulus to cause alterations in brain glucose transport that could be responsible for the defects of hormone counterregulation to subsequent hypoglycemia presently observed.

5.4.4 Why might SSTR2 antagonism not have as great of an effect on recurrent hypoglycemia as acute hypoglycemia?

In Study 1, we consistently observed robust restoration of glucagon and corticosterone counterregulation to acute hypoglycemia in diabetic rats with SSTR2 antagonism. Although glucagon, corticosterone, and epinephrine responses in Study 2 are shown to be significantly increased or preserved, the magnitudes of these improvements with SSTR2 antagonist treatment are not as profound following recurrent hypoglycemia. One possibility to explain this difference of the glucagon response is that STZ-diabetic rats are more insulin resistant than human type 1 diabetes, and thus much greater doses of insulin must be used to induce hypoglycemia in these animal experiments than is usually used in insulin-treatment of human diabetes. Thus following recurrent hypoglycemia, the sensitivity of diabetic pancreatic α-cells to the suppressive effects of exogenously administered insulin becomes so overwhelming that disinhibition of somatostatin’s effect on the α-cell is ineffective at stimulating glucagon release to hypoglycemia. Peak absolute plasma levels of insulin were similar in both Study 1 (D: 93.2 ± 7.2 ng/mL, D+SSTR2a: 107.9 ± 14.1 ng/mL; Table 4-3) and Study 2 (ExptD2-Ctrl: 71.9±18.7 ng/mL, ExptD2-SSTR2a:...
75.7±12.3ng/mL; Table 5-4), and even exhibited a tendency to be greater in the acute hypoglycemia experiment of Study 1; thus it is likely that the sensitivity to insulin, rather than the absolute amount of circulating insulin, is the more important factor for suppressing glucagon release. We have previously demonstrated that chronic, moderate insulin treatment administered for the purpose of lessening insulin resistance in STZ-diabetic rats helps to improve sensitivity to insulin in part, but that these rats still necessitated more insulin to induce hypoglycemia than non-diabetic rats, despite co-administration of phloridzin (641).

Another explanation for the diminished capacity of SSTR2 antagonist to fully enhance hormone responses following recurrent hypoglycemia is that different mechanisms regulate the defects of counterregulatory hormone responses to acute versus recurrent hypoglycemia. Thus, while SSTR2 blockade can acutely augment glucagon and corticosterone in diabetic rats, presumably because elevated pancreatic and/or circulating somatostatin, it is possible that elevated somatostatin does not contribute to the changes in neurocircuitry and central mechanisms that are altered with recurrent hypoglycemia. As discussed in part above, some of these mechanisms include, but are not limited to: (i) alterations of key brain regions responsible for glucose-sensing (245;660;670); (ii) alterations of mechanisms responsible for relaying counterregulatory responses (197;220;641;661;671); and/or (iii) a compensatory increase in glucose and/or other fuel uptake by the brain (38;178;672). Despite the very modest improvements in hormone counterregulation attributable to SSTR2 antagonist administration, it is noteworthy to emphasize that alleviation of the effect somatostatin via SSTR2 resulted in a remarkable amelioration of hypoglycemia in recurrently hypoglycemic diabetic rats.
5.5 Study 2 Conclusions

We provide support that recurrent hypoglycemia in diabetic rats markedly attenuates glucagon and epinephrine responses, as compared to the acute responses in Study 1, to subsequent hypoglycemia. We demonstrate that hypoglycemia can be ameliorated by SSTR2 antagonism following recurrent hypoglycemia in diabetic rats by lessening the depth and duration of hypoglycemia, and this glycemic rescue may be attributable in part to enhanced glucagon, epinephrine, and/or corticosterone release. We hypothesize that somatostatin may act via SSTR2 at various sites – pancreas, adrenal, brain, and/or pituitary, to regulate counterregulation in diabetes but do not exclude other mechanisms of impairment. Further investigation is necessary to further elucidate the mechanisms by which euglycemia is restored by inhibiting the action of somatostatin. By minimizing the risk of hypoglycemia, intensive insulin treatment would be more effective, leading to a lower incidence of diabetic complications and decreasing the stress associated with the anxiety of becoming hypoglycemic, consequently improving the quality of life for diabetic patients.
**Figure 5-1.** Study 2 protocol design.

**days -7 to 0:** acclimatization of male Sprague-Dawley rats (300-400 g)

**day 0:** Induction of diabetes (Ip STZ, 65 mg/kg)

**day 14:** catheterization surgery (L carotid artery & R jugular vein)

**day 18 to 20:** recurrent hypoglycemia treatment (5 episodes over 3 days)

**day 21:**

ExptD1: hypoglycemia without SSTR2 antagonist
(purpose: to determine minimum amount of insulin required to induce hypoglycemia in a given rat)

<table>
<thead>
<tr>
<th>saline infusion</th>
<th>saline infusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>hypoglycemia: 3.0 ± 1.0 mM</td>
<td>hypoglycemia: 3.0 ± 1.0 mM</td>
</tr>
<tr>
<td>Insulin: <strong>IV bolus</strong> (10 U/kg) + monitored infusion (50 mU/kg/min)</td>
<td>Insulin: <strong>IV bolus</strong> (10 U/kg) + monitored infusion (50 mU/kg/min)</td>
</tr>
<tr>
<td>60 min</td>
<td>240 min</td>
</tr>
<tr>
<td>n=13</td>
<td>n=7</td>
</tr>
</tbody>
</table>

**day 22:**

ExptD2 treatment: hypoglycemia with SSTR2 antagonist

<table>
<thead>
<tr>
<th>SSTR2a Infusion</th>
<th>saline infusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>hypoglycemia</td>
<td>hypoglycemia</td>
</tr>
<tr>
<td><strong>same insulin regime as experimental day 1</strong></td>
<td><strong>same insulin regime as experimental day 1</strong></td>
</tr>
<tr>
<td>60 min</td>
<td>240 min</td>
</tr>
</tbody>
</table>
Figure 5-2. Pilot study: A) Glycemia during 2 consecutive days of hypoglycemia (i.e. without prior recurrent hypoglycemia). B) Plasma glucagon levels during 2 consecutive days of hypoglycemia. Total average insulin administered = 4.8 U s.c. Data are represented as mean ± SEM.

A.  

B.
Figure 5-3. Pilot study: A) Glycemia during 2 consecutive days of hypoglycemia (i.e. without prior recurrent hypoglycemia). B) Plasma glucagon levels during 2 consecutive days of hypoglycemia. Insulin administered via i.v. bolus + infusion. Data are represented as mean ± SEM.
Figure 5-4. Blood glucose levels during recurrent hypoglycemia treatment (5 episodes over 3 days) in rats that will later be divided into SSTR2 antagonist-treatment (saline-SSTR2a; dark grey squares (■)) and control (saline-saline; light grey circles (●)) groups for hypoglycemia experiments. Data are represented as mean ± SEM.
Figure 5-5. Blood glucose levels during hypoglycemia experiments in saline-SSTR2a treatment group. ExptD1: black squares (■), and ExptD2: white squares (□). Data are represented as means ± SEM. An enlarged scale of the period of interest (i.e. euglycemia and hypoglycemia) is presented in Figure 5-7A. †P<0.02 vs. ExptD1
Figure 5-6. Blood glucose levels during hypoglycemia experiments in saline-saline control group. ExptD1: black circles (●), and ExptD2: white circles (○). Data are represented as means ± SEM. An enlarged scale of the period of interest (i.e. euglycemia and hypoglycemia) is presented in Figure 5-7B.
Figure 5-7. Enlarged scale showing blood glucose levels during hypoglycemia experiments in the A) saline-SSTR2a treatment group (solid bars) and B) saline-control group (hashed bars) on ExptD1 (black) and ExptD2 (white). Inset graphs represent extent of hypoglycemia and are calculated as area under the curve of glycemia <4.0 mM. Data are represented as means ± SEM. *P<0.001 vs. ExptD1. †P<0.02 vs. ExptD1
Figure 5-8. Plasma glucagon during ExptD1 (black) and ExptD2 (white) in A) saline-SSTR2a treatment (solid bars); B) saline-SSTR2a treatment with rats that did not experience hypoglycemia omitted; and C) saline-saline control groups (hashed bars). Data are represented as mean ± SEM. *P<0.04 vs. ExptD1.
**Figure 5-9.** Plasma epinephrine during ExptD1 (black) and ExptD2 (white) in A) saline-SSTR2a treatment (solid bars); B) saline-SSTR2a treatment with rats that did not experience hypoglycemia omitted; and C) saline-saline control groups (hashed bars). Data are represented as mean ± SEM. * P<0.03 vs. ExptD1.
Figure 5-10. Plasma corticosterone during ExptD1 (black) and ExptD2 (white) in A) saline-SSTR2a treatment (solid bars); B) saline-SSTR2a treatment with rats that did not experience hypoglycemia omitted; and C) saline-saline control groups (hashed bars). Data are represented as mean ± SEM. * P<0.04 vs. ExptD1.
Figure 5-11. Plasma norepinephrine during ExptD1 (black) and ExptD2 (white) in A) saline-SSTR2a treatment (solid bars); B) saline-SSTR2a treatment with rats that did not experience hypoglycemia omitted; and C) saline-saline control groups (hashed bars). Data are represented as mean ± SEM.
Figure 5-12. Comparison of peak incremental hormone responses to acute hypoglycemia (Study 1; solid bars) and recurrent hypoglycemia (RH) (Study 2; speckled bars) in control (Ctrl; black) and SSTR2 antagonist-treatment (SSTR2a; white) groups. A) Plasma glucagon; B) plasma epinephrine; C) plasma corticosterone. Data are represented as mean ± SEM. *P<0.003 vs. Acute-Ctrl. †P<0.05 vs. RH-Ctrl. ‡P<0.03 vs. Acute-Ctrl.
**Table 5-1.** Metabolic parameters for rats that underwent recurrent hypoglycemia treatment. Fed blood glucose, body weight, and food intake were measured daily, and the table shows the mean ± SEM of values averaged over the last week before commencement of recurrent hypoglycemia treatment in rats that would later be divided into SSTR2a-treatment and control groups for hypoglycemia experiments. Daily blood glucose measurements were obtained using a glucometer.

<table>
<thead>
<tr>
<th></th>
<th>Treatment group (saline-SSTR2a)</th>
<th>Control group (saline-saline)</th>
</tr>
</thead>
<tbody>
<tr>
<td>fed blood glucose (mM)</td>
<td>25.3 ± 0.9</td>
<td>23.6 ± 2.1</td>
</tr>
<tr>
<td>body weight (g)</td>
<td>365 ± 6</td>
<td>372 ± 11</td>
</tr>
<tr>
<td>food intake (g)</td>
<td>39 ± 1</td>
<td>37 ± 2</td>
</tr>
</tbody>
</table>
Table 5-2. Body weight and amount of insulin administered via iv bolus and iv infusion, and total insulin administered in both groups on both days. 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 hypoglycemia was neared. This volume of insulin infusion was recorded on ExptD1 and repeated on ExptD2. Data are represented as mean ± SEM.

<table>
<thead>
<tr>
<th></th>
<th>Treatment group (saline-SSTR2a)</th>
<th>Control group (saline-saline)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ExptD1</td>
<td>ExptD2</td>
</tr>
<tr>
<td>body weight (g)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>initial glycemia (mM)</td>
<td>25.5 ± 1.7</td>
<td>24.1 ± 1.9</td>
</tr>
<tr>
<td>insulin via iv bolus (U)</td>
<td>3.24 ± 0.27</td>
<td>3.26 ± 0.29</td>
</tr>
<tr>
<td>insulin via iv infusion (U)</td>
<td>0.88 ± 0.11</td>
<td>0.90 ± 0.11</td>
</tr>
<tr>
<td>total insulin administered (U)</td>
<td>4.12 ± 0.31</td>
<td>4.16 ± 0.34</td>
</tr>
</tbody>
</table>
Table 5-3. Basal plasma hormone levels on Experimental Day 1 (ExptD1) and Experimental Day 2 (ExptD2) in rats that underwent SSTR2a-treated and control hypoglycemia experiments. These represent basal hormone levels after 5 episodes of recurrent hypoglycemia and a partial overnight-fast. Data are represented as mean ± SEM. * P<0.05 vs. ExptD1

<table>
<thead>
<tr>
<th>Treatment group (saline-SSTR2a)</th>
<th>Control group (saline-saline)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ExptD1</td>
</tr>
<tr>
<td>glucagon (pg/mL)</td>
<td>58 ± 5</td>
</tr>
<tr>
<td>epinephrine (pg/mL)</td>
<td>106 ± 17</td>
</tr>
<tr>
<td>corticosterone (ng/mL)</td>
<td>110 ± 20</td>
</tr>
<tr>
<td>norepinephrine (pg/mL)</td>
<td>326 ± 77</td>
</tr>
<tr>
<td>insulin (ng/mL)</td>
<td>0.98 ± 0.2</td>
</tr>
</tbody>
</table>


**Table 5-4.** Plasma insulin levels (ng/mL) during ExptD1 and ExptD2 in saline-SSTR2a treatment and saline-saline control groups. Insulin was administered as an i.v. bolus followed by i.v. infusion after samples were obtained at the 0 time point. Infusions were stopped at the experimenter’s discretion (usually between 30-120 min) but kept consistent for the same rat on both experimental days. Data are represented as mean ± SEM.

<table>
<thead>
<tr>
<th></th>
<th>-60 ± 0.15</th>
<th>0 ± 0.17</th>
<th>60 ± 15.71</th>
<th>180 ± 0.09</th>
<th>240 ± 0.05</th>
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<tr>
<td><strong>ExptD1</strong></td>
<td>0.98</td>
<td>0.90</td>
<td>96.71</td>
<td>1.13</td>
<td>0.70</td>
</tr>
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<td><strong>saline-SSTR2a</strong></td>
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<td></td>
<td></td>
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</tr>
<tr>
<td><strong>ExptD2</strong></td>
<td>0.98</td>
<td>1.03</td>
<td>75.71</td>
<td>1.03</td>
<td>0.75</td>
</tr>
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<td><strong>saline-saline</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>ExptD1</strong></td>
<td>1.00</td>
<td>1.02</td>
<td>89.27</td>
<td>1.43</td>
<td>0.63</td>
</tr>
<tr>
<td><strong>ExptD2</strong></td>
<td>0.97</td>
<td>0.93</td>
<td>71.93</td>
<td>1.32</td>
<td>0.60</td>
</tr>
</tbody>
</table>
6 Study Summaries and Limitations

Hypoglycemia remains an obstacle and limitation to stringent glycemic control in diabetes (4;161). The result of β-cell failure in type 1 diabetes and advanced type 2 diabetes causes the loss of the first two key defenses against a fall in blood glucose levels: the decrement of insulin and the corresponding α-cell response to release glucagon (172). Since glucagon counterregulation normally plays the primary role in promoting glycemic recovery from hypoglycemia (510), such that even increases of epinephrine cannot fully compensate for the absence of glucagon on hepatic glucose production during hypoglycemia (62), it is of great clinical relevance to discover methods to improve and restore glucagon counterregulation to hypoglycemia in diabetes. Two main theories underlay the glucagon deficiency in the present thesis: that in diabetes, the α-cell becomes more sensitive to the inhibitory effects of exogenous insulin, and that excessive somatostatin further inhibits the ability of the diabetic α-cell to release glucagon via SSTR2 agonism during hypoglycemia. Because somatostatin also has been shown to suppress stress-induced epinephrine and cortisol/corticosterone release (169;528;628;652;654;656), and because SSTR2 are present in tissues that store or regulate epinephrine and cortisol/corticosterone secretion (373;378;379;390;397-400), we also hypothesized that SSTR2 blockade would enhance the release of these counterregulatory hormones to hypoglycemia. The main objectives of this thesis were: 1) to evaluate a means to improve the counterregulatory response to hypoglycemia in a diabetic animal model by use of a SSTR2 antagonist, and 2) to assess if
counterregulatory hormone improvements could be extended and have a biological effect to ameliorate hypoglycemia after exposure to recurrent hypoglycemia.

6.1 Summary of Study 1
Data from the present study demonstrate that diabetic rats had markedly attenuated glucagon and corticosterone responses to hypoglycemia as compared to non-diabetic rats, which is in agreement with our previous studies (205). We demonstrate that glucagon and corticosterone counterregulatory hormone responses to hypoglycemia can be restored in diabetic rats with specific antagonism of the SSTR2 using both high (10 U/kg) and low (5 U/kg) doses of insulin to induce hypoglycemia. We attribute these defects in part to elevated pancreatic and circulating somatostatin levels which were increased in diabetic rats following hypoglycemia. SSTR2 antagonist given to non-diabetic rats, in which somatostatin levels were the same as non-diabetic controls, did not increase glucagon or corticosterone responses, which further provides support for the contribution of augmented somatostatin. During basal conditions in the absence of insulin, this SSTR2 antagonist does not elicit hyperglycemia or substantial elevations in counterregulatory hormones, which is important if this antagonist is potentially considered for therapeutic use in humans. Under conditions of lower dose insulin injection, SSTR2 antagonism reduced the glucose requirement during hypoglycemic clamp, which is consistent with improved glucagon and corticosterone counterregulation and is indicative of a lower risk of hypoglycemia. In these 3-week STZ-diabetic rats, epinephrine and norepinephrine counterregulation to hypoglycemia induced with both high and low doses of insulin were similar in both non-diabetic and diabetic rats; thus, these rats did not demonstrate a defect in catecholamine responses to hypoglycemia. Epinephrine, norepinephrine, and growth hormone levels were not affected by SSTR2
administration. Despite improvements of glucagon and corticosterone to hypoglycemia, expression of gluconeogenic enzymes PEPCK1 and G6Pase were unaltered.

### 6.2 Summary of Study 2

In light of the evidence from Study 1 that SSTR2 antagonism could normalize glucagon and corticosterone response to hypoglycemia, we designed a set of experiments to determine whether such improvements in hormone responses could have a biological effect and improve recovery from hypoglycemia in a model with established counterregulatory defects: recurrent hypoglycemia. In these experiments, we first subjected diabetic rats to 5 episodes of hypoglycemia. Subsequently, we compared the responses of each rat over two experimental days. Much care was taken to control for similar baseline metabolic parameters, such as initial glycemia, body weight, and overnight food intake, so that meaningful and relevant comparisons between the two experimental days could be made. On the first experimental day (ExptD1), we determined the minimum amount of insulin necessary to induce hypoglycemia at 3.0 mM in a given rat, which was necessary since diabetic rats have varying insulin sensitivities. On the second experimental day (ExptD2), we used the same dose of insulin in the same rat and compared its glycemic and hormone responses to ExptD1. We included two groups in this study: a treatment group which received SSTR2 antagonist on ExptD2, and a control group which did not receive SSTR2 antagonist on ExptD2. By comparison with Study 1, we demonstrated that recurrent hypoglycemia further reduced the attenuated glucagon response, and markedly reduced the epinephrine response, to subsequent hypoglycemia in diabetic rats. Our most striking finding was that hypoglycemia can be ameliorated by SSTR2 antagonism in diabetic rats, despite the marked attenuation of glucagon and epinephrine caused by recurrent
hypoglycemia. By blocking the actions of somatostatin on SSTR2, the depth and duration of hypoglycemia were significantly lessened, and a quicker recovery to euglycemia ensued in animals, despite prior exposure to repeated hypoglycemia. This amelioration of hypoglycemia may be due, at least in part, to enhanced glucagon release and to improvements in epinephrine and corticosterone counterregulation which are observed in the SSTR2 antagonist treatment group. Norepinephrine levels were not affected by recurrent hypoglycemia or by SSTR2 antagonist treatment.

6.3 General discussion

Our results suggest an important role for increased pancreatic, and possibly circulating, somatostatin in defective glucagon, corticosterone, and/or epinephrine counterregulation in diabetes but do not exclude other mechanisms of impairment. From our present findings, it is hypothesized that selective SSTR2 blockade at the levels of the brain, pituitary, pancreas, and adrenal gland during hypoglycemia may help to reduce the severity, duration, or onset of hypoglycemia by enhancing the release of counterregulatory hormones such as glucagon, corticosterone, and epinephrine.

6.3.1 Limitations of the present study

The main limitation is that a precise mechanism of action of the SSTR2 antagonist could not be delineated from the work of this thesis. From the data presented in this thesis, we can suggest that SSTR2 antagonism plays a role in restoring glucagon and corticosterone counterregulation to acute hypoglycemia, and has some effect to improve glucagon, epinephrine, and corticosterone responses following recurrent hypoglycemia, but we have yet to delineate the precise mechanism by which this occurs. These impairments in
hormone responses can occur at the tissue level (pancreatic islets for glucagon, adrenal cortex for corticosterone, or adrenal medulla for epinephrine), via the peripheral nerves, or at the level of the brain or pituitary. We suggest that increased pancreatic somatostatin, and potentially increased circulating somatostatin, can affect local and central control of counterregulatory hormone release. With respect to deficient glucagon counterregulation, we hypothesize that despite the fact that somatostatin may not be excessive in certain models of diabetes, antagonism of somatostatin’s suppressive actions may still be beneficial since diabetic α-cells are already more sensitive to the inhibition of insulin. With respect to impaired corticosterone counterregulation, we hypothesize that an additional alleviation of suppression of corticosterone release may improve this response to hypoglycemia. Our laboratory has previously demonstrated that in diabetes, corticosterone release to hypoglycemia is attenuated by suppression on the HPA axis (202;220;641;673). Although we did not delineate a role for somatostatin on this inhibition, we presently hypothesize that somatostatin may inhibit activation of the HPA axis since somatostatin is known to inhibit release of neurotransmitters and hormones involved in the HPA response, namely norepinephrine, CRH, and ACTH. However, the limitation remains that studies to assess the direct effect of SSTR2 blockade in the hypothalamic PVN (which could be achieved by stereotaxic microinjection of the SSTR2 antagonist) or in corticotropes of the anterior pituitary were not performed. Measurement of plasma ACTH could also provide an indication as to whether inhibition of somatostatin involved an effect at the level of the anterior pituitary. The anterior pituitary is not protected by the blood-brain-barrier, and peripheral administration of the SSTR2 antagonist could theoretically bind with SSTR2 in the anterior pituitary. Thus, if the SSTR2-mediated improvements in corticosterone counterregulation are accompanied by increased ACTH levels, this would indicate a direct
effect of disinhibition at the level of the corticotrope or suggest an involvement of a central mechanism.

In addition, another limitation is that we did not measure glucose turnover in these studies. Precise and accurate measurements of glucose turnover could have helped us to determine whether the enhancement of counterregulatory hormones and improvement in hypoglycemic recovery associated with SSTR2 blockade had an effect on glucose production, glucose utilization, or both. Further investigation is therefore necessary to elucidate the mechanism by which hormone counterregulation is improved and euglycemia is restored via inhibiting the action of somatostatin via SSTR2.

6.3.2 Hypothesizing a mechanism of action: CNS effects?

Our initial hypothesis was that the concomitant elevation of pancreatic somatostatin and heightened sensitivity of diabetic α-cells to exogenous insulin contributed to the impairment of the glucagon response to hypoglycemia in diabetes. This, of course, cannot explain the improvements of corticosterone and epinephrine counterregulation with SSTR2 disinhibition. In the Discussions of Study 1 and Study 2, several potential mechanisms were addressed that may underlie SSTR2-mediated improvements to glucose counterregulation. In addition to these, this section will briefly propose possible roles of SSTR2 antagonism within the brain that could regulate counterregulatory hormone responses.

There is vast, compelling evidence dating back to early studies of Claude Bernard in the 19th century in which he demonstrated that punctures of the fourth cerebral ventricle rendered dogs glycosuric (674). Recent reviews (13;14;193) on brain glucosensing and glucose counterregulation also irrevocably demonstrate the importance of the central
nervous system on regulating glucose homeostasis. Initiation of counterregulatory hormone responses in the central nervous system involves: (i) afferents that detect changes in glucose levels in the brain and/or periphery; (ii) integration and relay of signals within specific brain regions; and (iii) efferent pathways from integration centres in the brain that project as autonomic signals to the body to maintain glucose homeostasis (13;14;193).

Interestingly, many of these brain regions that comprise the neural networks that regulate hypoglycemic counterregulation also are rich in somatostatin and its receptors. This is not surprising since within the brain, somatostatin serves as a neurotransmitter. Yet it allows us to speculate: could alterations in brain somatostatin action as a result of diabetes and/or recurrent hypoglycemia contribute to the impairments of hypoglycemic counterregulation?

In addition to mechanisms within the pancreas responsible for glucagon release in response to hypoglycemia (76), glucagon counterregulation is also regulated centrally. Buijs et al. used a pseudorabies virus as a retrograde transsynaptic tracer to elucidate the neuronal parasympathetic and sympathetic efferent pathways that control pancreatic hormone secretion (99). Buijs et al. identified a sympathetic pathways involving (i) neurons of the ventromedial hypothalamus (VMH), arcuate nucleus (ARC), central amygdala, bed nucleus of the stria terminalis (BNST), suprachiasmatic nucleus (SCN), and medial preoptic area (MPO) projecting to the paraventricular nucleus (PVN) and hypothalamic zona incerta (ZI), which in conjunction with a projection from the lateral hypothalamus (LHA) project to the intermediolateral column (IML) of the spinal cord; and (ii) neurons of the nucleus of the solitary tract (NTS) projecting to the locus coeruleus (LC) of the brain stem, which subsequently joins a projection from the prefrontal cortex, or from the NTS to the rostroventrolateral medulla (RVLM) to project to the IML. Both of these neuronal pathways subsequently projecting from the IML of the spinal cord provides the sympathetic efferent to
the pancreas to stimulate glucagon release (99). In addition, in a recent review, Watts and Donovan summarized the interactions between networks involving the periphery, hindbrain, and hypothalamic components involved with hypoglycemic counterregulation (14). In brief, glucose sensors are located in the portal/mesenteric vein (PMV), hindbrain, and hypothalamic nuclei (LHA, ARC, and VMH). These signals are then integrated in networks involving the hindbrain and hypothalamus. The hindbrain and hypothalamic integrators then activate autonomic efferent neurons to stimulate glucagon and catecholamine release, or via a neuroendocrine route the PVN stimulates glucocorticoid release (14). In his review, Cryer has also indicated key brain regions that participate in the mechanisms of hypoglycemia-associated autonomic failure, which include the prefrontal cortex, the dorsal midline thalamus, hypothalamus, hippocampus, brain stem, and amygdala (4).

These recent reviews identify key brain areas involved with hypoglycemic counterregulation. Within the brain, somatostatin-immunoreactivity is found in many neurons. As reviewed by Viollet et al., somatostatinergic neurons are highly detected in the mediobasal hypothalamus, median eminence, amygdala, hippocampus, cerebral cortex, and brainstem (292). In addition to short projections that act within a local area, somatostatinergic neurons can also project to other target areas, and several examples include: amygdala → parabrachial nucleus (PBN), NTS, and dorsal motor nucleus of the vagus (DMNV); periventricular nucleus → median eminence, ARC, habenula, and LC; PBN → thalamus; and NTS → PVN, PBN. SSTR2 expression has been reported in hypothalamic ARC, PVN, and VMH (420;424;426), medial habenular nucleus of the thalamus (420), cerebral cortex, hippocampus, LC, basolateral amygdala (BLA), NTS, PBN, and spinal cord (419;420;425). In light of the fact that several SSTR2-expressing brain regions endogenously are targeted by somatostatinergic neurons, and that these regions also play a role in glucose-detection
and/or regulation of the counterregulatory responses, it would be of interest to evaluate the
effect of specific SSTR2 antagonism on glucose-regulatory neuronal populations. This could
be achieved by inhibiting these receptors during hypoglycemia by directly administering the
SSTR2 antagonist to specific brain regions or by silencing the SSTR2 gene at specific brain
nuclei via viral gene delivery. Also, it would be interesting to determine whether these
glucose-regulatory neurons are affected by diabetes and/or hypoglycemia. This could
provide new insights for the role of somatostatin in diabetic hypoglycemic counterregulation.

6.3.2.1 Does peripherally administered SSTR2 antagonist enter the brain?

We are not aware at the present time of any reports whether our SSTR2 antagonist can
enter the brain when administered peripherally via an intravenous route. CTAP, a cyclic
octapeptide with the structure D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH2 is a μ-opiod
receptor antagonist that has been reported to cross the blood-brain-barrier (562).
However, the majority of literature, however, suggests that a compound like our cyclized
octapeptide antagonist (see Figure 1-2 for structure) would likely not cross the blood-brain-
barrier (675). Octreotide, an octapeptide somatostatin analogue, is unlikely to cross the
blood-brain-barrier in any significant amounts when peripherally administered (676).
Furthermore, SSTR3 octapeptide antagonists cyclized via a disulfide bridge have been
reported to be very poor at crossing the blood-brain-barrier (677). In view of these reports,
our peripherally infused SSTR2 antagonist may not have entered the brain to have any
central effects. Further investigation is needed to more precisely determine whether this
compound could enter the brain. The MDCK-MDR1 cell line is an established in vitro model
of the blood-brain-barrier and can be used to test the permeation properties of peptides
(678).
6.3.3 Brief evaluation of tachyphylactic effects of SSTR2 antagonism

It is known that G-protein coupled receptors (GPCRs), including SSTRs, share a common property whereby they regulate their own responsiveness to continued agonist exposure (679). For example, GPCRs become desensitized after acute treatment to agonists. Three main steps are responsible for this: 1) uncoupling of the GPCR from G proteins; 2) receptor internalization via the phosphorylation of the C-terminal tail and intracellular loops by second-messenger-activation or GPCR-kinases, which has been demonstrated in rat pituitary and islet tumour cells; and 3) receptor downregulation. SSTR2, for example couples with adenylyl cyclase via inhibitory G proteins when activated by somatostatin and undergoes endocytosis via clathrin-coated pits (680). However, GPCRs also become upregulated after prolonged agonist exposure (679). This is associated with: 1) the active process of ligand-induced receptor recruitment from a preexisting cytoplasmic pool; 2) does not require new protein synthesis; and 3) depends on molecular signals on the C-terminal tail. This has also been observed in receptors for somatostatin (681). These findings are relevant for agonist exposure. However, it remains to be seen if the same applies for antagonist exposure. For our SSTR2 antagonist used in this study, in which the peptide ligand binds to the SSTR2 without inducing the same intracellular responses as somatostatin would, it is not known how prolonged exposure would affect receptor expression on target tissues, such as pancreatic α-cells, the brain, or adrenal glands. There is no consensus in literature as to whether antagonists initiate internalization of GPCRs. Peptide receptor antagonists for cholecystokinin (682) and neuropeptide Y (683), for example, have been reported to stimulate receptor internalization. One study demonstrated that SSTR agonists, but not antagonists internalized SSTR2 (680). Another study likewise demonstrated that antagonists for SSTR2 or SSTR3 did not affect internalization in human embryonic kidney
cells (684), but studies for our specific antagonist and its result in α-cells are still required to elucidate the long-term efficacy of this peptide.

6.3.4 Potential side effects of SSTR2 antagonism

Since receptors for somatostatin are expressed ubiquitously throughout the body, it is important to target the particular tissue – and specifically the cell type – in which a pharmacological compound is to take effect. In particular, we initially sought to target SSTR2 because of its mediation of somatostatin’s inhibitory effect on α-cell glucagon release. Using an antagonist with high selectivity, binding affinity, and biological activity is of key importance, and the peptide antagonist that was used in this study offered these characteristics (509). However, although most tissues tend to show preferential expression for particular SSTR subtypes, SSTR2 is still expressed in high levels in several areas. These areas include the stomach, the cerebral cortex, pancreatic α-cell, and adrenal medulla. In the pituitary, SSTR5 has the highest level of expression, followed by SSTR2. The high expression of SSTR2 on α-cells and the adrenal medulla is of course favourable for our studies in glucagon and epinephrine counterregulation. These other tissues/cell types are mentioned here to assess potential side effects of SSTR2 antagonist administration during our experiments.

Although pancreatic β-cells in rodents exclusively express SSTR5 and not SSTR2 (387), high levels of SSTR2 antagonist without sufficient competition by native somatostatin or with saturation of SSTR2 on α-cells may lead to non-specific binding with SSTR5. This would lead to enhanced insulin secretion from functional β-cells, including those in non-diabetic subjects or possibly in non-advanced type 2 diabetes. This may explain why diminished glucagon responses to hypoglycemia were observed in our studies of SSTR2 antagonist in non-diabetic rats. This would not be a concern, however, in type 1 diabetes or likely not in
STZ-diabetes where the majority of \( \beta \)-cells are destroyed. Furthermore, although human \( \beta \)-cells predominantly express SSTR1 and SSTR5, SSTR2 is expressed in up to 25% of human \( \beta \)-cells, which may suggest a greater potential side effect of SSTR2 antagonist in promoting insulin release. Thus, patients with type 2 diabetes who still have insulin secretory capacity should avoid SSTR2 antagonist as a therapy to prevent hypoglycemia.

In the stomach, SSTR2 has been reported as the main subtype through which somatostatin inhibits gastrin-stimulated gastric acid secretion both through studies using SSTR2 peptide analogs (685) and SSTR2 knockout mice (686). A study using the same SSTR2 antagonist in this present work demonstrated in non-diabetic rats that SSTR2 antagonism blocked the inhibitory effect of somatostatin on gastrin-stimulated release. At a dose which we used in our pilot studies (1500 nmol/kg/h), the SSTR2 antagonist likewise inhibited the suppressive effect of GLP-1 on gastrin-stimulated gastric acid output (508). However, a recent study reported normal circulating levels of gastric and unaltered gastric acid output in SSTR2 knockout mice (687), which suggests that blocking the effects mediated via SSTR2 may not necessarily affect gastric function. However, it remains possible that in our studies, stimulated-gastric acid output could have been increased in our rats. Other gut effects mediated by somatostatin action via SSTR2, such as suppression of intestinal mucosal cell proliferation and colonic motility, may also be inhibited by SSTR2 antagonism. Although glucose absorption was shown to be inhibited by somatostatin (688), the receptor subtype by which this action is mediated was not determined.

In the brain, somatostatin functions as a neurotransmitter contributing to cognitive, locomotor, sensory, and autonomic activities (271). SSTR1 is considered by many reviews to be more vastly distributed and highly expressed than other receptor subtypes, and is often found in higher levels than SSTR2 in areas such as the cerebral cortex and
hippocampus where SSTR2 is considered to be highly expressed (271;378;420).

Somatostatin was initially known for its effect to suppress growth hormone secretion (267). Somatostatin produced in the periventricular nucleus (where hypothalamic somatostatin is greatest (271)) and released from the median eminence directly inhibits growth hormone release from somatotrophes of the anterior pituitary via SSTR2 and SSTR5 (292;431). SSTR5 is the main subtype expressed throughout the pituitary, followed by SSTR2 (380). There is also evidence that SSTR1 is also implicated in the control of growth hormone release (433). Whereas SSTR2 antagonist disinhibition to stimulate growth hormone secretion would be favourable during hypoglycemia, this was not observed in our present findings. However, it remains to be determined whether prolonged treatment with SSTR2 antagonist, or blood sampling at more frequent intervals, could stimulate growth hormone release. Presently, we have no direct data to report whether SSTR2 antagonist stimulated anterior pituitary secretion of adrenocorticotrophic hormone (ACTH), but our improved corticosterone data with SSTR2 antagonist treatment could support this. Other studies have demonstrated an inhibitory effect of somatostatin on ACTH release (294) and the presence of SSTR2 on ACTH cells in the anterior pituitary (561). Other pituitary hormones, such as thyroid stimulating hormone and adrenocorticotropic hormone, may also be altered by SSTR2 antagonism (290).
7 Future Directions

7.1 Delineating a mechanism of action for SSTR2 antagonism

To address the main shortcoming of this project, which is a lack of mechanistic explanation for the SSTR2-antagonism mediated improvements in glucose counterregulation, an important future study would be to delineate a mechanism by which SSTR2 blockade acts to: (i) improve hormone counterregulation to acute hypoglycemia; (ii) more effectively restore low blood glucose back to euglycemia. For the latter, tracer experiments using traditional hyperinsulinemic-hypoglycemic clamp techniques can provide glucose turnover data which may provide insights as to whether somatostatin affects glucose production or uptake during hypoglycemia. This is important since we have previously shown that peak glucose production was decreased during hypoglycemia in diabetic rats (205). An improvement in net glucose production is essential since the overall purpose in improving counterregulation is to restore plasma glucose levels in diabetic patients suffering from hypoglycemia. However, despite the unspecified etiology of the effect, a significant improvement in glycemic recovery was demonstrated by SSTR2 inhibition, and it is often the overall effect of a pharmacological compound that garners the interest and attention of patients.

To determine whether normalized glucagon and corticosterone counterregulation was mediated via a central nervous system effect, SSTR2 antagonist can be
intracerebroventricularly administrated during hypoglycemia in diabetic rats. Finally, evaluating the counterregulatory hormone responses to hypoglycemia in genetic animal models with tissue-specific SSTR2 knockout can also help to elucidate mechanisms of somatostatin action.

7.2 Testing the efficacy of SSTR2 antagonism in other diabetic models

In light of the fact that certain diabetic rodent models, such as the BB rat, which do not have increased pancreatic somatostatin, but do have elevated circulating somatostatin (537;689) do show impairments in the counterregulatory hormone responses to insulin-induced hypoglycemia (263;542;543), it is worthwhile to investigate the efficacy of SSTR2 antagonism in this model of experimental diabetes to determine the contribution of excessive pancreatic somatostatin on glucagon counterregulation in diabetes. It is unknown why BB rats do not have increased pancreatic somatostatin, unlike other experimental rat models of diabetes. An earlier hypothesis suggested that BB rats had “more severe islet destruction” than STZ-diabetic rats (537). In addition, it has been demonstrated that insulin treatment of STZ-diabetic rats can normalize, at least in part, pancreatic and circulating somatostatin levels, yet insulin-treated diabetic animals do not have fully normalized hormone counterregulation to hypoglycemia (174;202;641). Thus, performing similar hypoglycemia studies with and without SSTR2 antagonist in insulin-treated rats will allow us to determine whether improvements in diabetic hyperglycemia and α-cell sensitivity may alter the effectiveness of SSTR2 antagonism during hypoglycemia. Insulin-treated diabetic subjects may also be less insulin resistant, which would allow us to use less insulin to induce hypoglycemia. This is important since excessively high insulin levels alter
counterregulation and glucose production. Insulin-treated diabetes is more clinically relevant since type 1 diabetic humans are insulin treated.

7.3 Effect of SSTR2 antagonism to non-hypoglycemia stimuli

It is presently unknown whether SSTR2 antagonism will enhance glucagon release in response to other stimuli known to trigger glucagon release, such as an amino acid-rich meal or exercise. Hypoglycemia and amino acids stimulate glucagon secretion by two different processes, the latter of which only can be counteracted by amylin (690). Exercise and hypoglycemia similarly stimulate glucagon via activation of the autonomic nervous system and neuroendocrine response (149). Unlike hypoglycemia, the glucagon response to amino-acid rich meals (691-694) and exercise (149;695-697) are preserved, or even exaggerated, in diabetes. Thus, studies designed to evaluate the effects of SSTR2 antagonist on other glucagon secretagogues will be of interest, and it may help to determine if this effect to enhance counterregulatory hormones is hypoglycemia-specific. Plasma glucagon could be measured in response to: i) a high protein meal, and ii) exercise with and without administration of the antagonist, in the presence or absence of elevated somatostatin, and in non-diabetic or diabetic subjects, to determine the stimulus-specificity of SSTR2 inhibition and the condition(s) in which it applies.

7.4 Interaction between glycemia, insulin, and somatostatin antagonism on glucagon release

Using perfused pancreata or isolated islets, the direct effect of the SSTR2 antagonist on glucagon release can be assessed with carefully controlled interactions between glycemia,
insulin, and somatostatin antagonism on glucagon release. These *ex vivo* or *in vitro* experiments have the benefit of clarifying the role of somatostatin antagonism in the pancreas more directly since *in vivo* experiments also involve many other hormonal and metabolic changes. Efendic et al. has shown previously that this SSTR2 antagonist can increase arginine-induced glucagon release in perfused pancreata at both 5.5 and 3.3 mM glucose concentrations in non-diabetic rats (392). However, those experiments did not answer the main question of whether SSTR2 antagonist can, in presence of insulin which inhibits α-cells, increase glucagon release in diabetic islets, which are often deprived of the tonic effect of insulin. These experiments can also assess the contribution of increased α-cell sensitivity to insulin as a result of a chronic lack of the presence of insulin on glucagon secretion to insulin-induced hypoglycemia. These experiments could also show whether the local effect on glucagon release is as marked as when SSTR2 antagonist is given peripherally.
8 Conclusions

8.1 Importance of contributions to current body of knowledge

Our laboratory first suggested 20 years ago that increased somatostatin in diabetes may contribute to the impaired glucagon response to hypoglycemia in diabetes (90). We hypothesized that the combined suppressive effects of excessive pancreatic somatostatin and increased sensitivity of diabetic α-cells to exogenously administered insulin contributes to defective glucagon counterregulation. The work in this thesis demonstrates for the first time that antagonizing the actions of somatostatin acting on SSTR2 can fully abolish this glucagon defect in diabetes during acute hypoglycemia. Thus, disinhibition of the diabetic α-cell from somatostatin, one of its key suppressing influences, can improve its secretory capacity in response to hypoglycemic stimulation. We also demonstrate that the attenuated corticosterone response in diabetic rats was significantly improved with SSTR2 blockade, an effect which at the present time can only be speculated to due to alleviation of somatostatin’s inhibitory release of corticosterone, or other regulators of its release directly at the adrenal cortex or indirectly mediated in the brain or pituitary in diabetes. These improvements of glucagon and corticosterone were observed to be diabetes-specific since SSTR2 antagonism in non-diabetic rats during hypoglycemia did not increase these hormone responses. Importantly, we also demonstrate that during basal conditions in the absence of insulin that SSTR2 antagonist treatment neither causes marked sustained elevations of counterregulatory hormones nor elicits undesired hyperglycemia in diabetic rats. In
addition, we provide novel evidence that SSTR2 antagonism can promote the recovery of hypoglycemia, presumably in part by improving glucagon, and preserving epinephrine and corticosterone, hormone responses in diabetic rats exposed to repeated episodes of hypoglycemia. This exciting and novel finding reveals that the depth and duration of hypoglycemia can be significantly be reduced and even prevented, even in diabetic subjects with marked counterregulatory defects. Taken together, these findings implicate an important role for increased pancreatic, and possibly circulating, somatostatin in defective hypoglycemic counterregulation in diabetes and do not discount other mechanisms of impairment, which have been discussed in this thesis.

8.2 Potential clinical contributions

The goal of this work was to develop a means to ameliorate hypoglycemia. This objective differs from the current hypoglycemia therapies, such as injectable glucagon and dextrose, which are reactionary measures as opposed to preventive therapies. Hypoglycemia that is particularly dangerous includes that which occurs undetected, such as during sleep and in diabetic children and adolescents. Reactionary therapies necessitate the recognition of hypoglycemia by the individual or the care giver, which would be difficult with hypoglycemic unawareness or during nocturnal hypoglycemia. A prophylactic solution involves administration of a therapeudeic prior to hypoglycemia onset so that the duration/severity of hypoglycemia is lessened or prevented entirely so that the patient does not become debilitated. This could involve administration of the SSTR2 antagonist with one’s insulin, particularly when the risk of hypoglycemia may be greater (i.e. at bedtime and/or after exercise). By minimizing the risk of hypoglycemia, intensive insulin treatment would be more effective, leading to a lower incidence of diabetic complications and decreasing the
stress associated with the anxiety of becoming hypoglycemic, consequently improving the quality of life for diabetic patients.
Chapter 9

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