Somatostatin Receptor Type 2 Antagonism Improves Glucagon Counter-Regulation in Biobreeding Diabetes-Prone Rats

By

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Impaired counterregulation during hypoglycemia in type 1 diabetes (T1D) is partly due to inadequate pancreatic islet alpha-cell glucagon secretion. We hypothesized that hypoglycemia can be prevented in autoimmune T1D by selective somatostatin receptor type 2 (SSTR2) antagonism of alpha cells to relieve SSTR2 inhibition, thereby increasing glucagon secretion. Diabetic biobreeding diabetes prone (BBDP) rats (D) vs non-diabetic BBDP (N) rats, underwent infusion of vehicle or SSTR2 antagonist (SSTR2a) during insulin-induced hypoglycaemia. D rats, treated with SSTR2a, needed little or no glucose to maintain hypoglycemia. To monitor real-time glucagon secretory response directly, we developed the technique of thin slices of the pancreas from D and N rats as well as
normal human pancreas, subjected to perifusion with vehicle vs SSTR2a. SSTR2a treatment enhanced glucagon secretion in N and D rats and human pancreas. We conclude that SSTR2 antagonism can enhance hypoglycemia-stimulated glucagon release sufficient to achieve normoglycemic control.
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Chapter 1 Introduction

1.1 Hypoglycemia

The American Diabetes Association Workgroup on Hypoglycemia (Cryer, 2005) defined hypoglycemia in diabetes as “all episodes of abnormally low plasma glucose concentration that expose the individual to potential harm.” They recommended that at a plasma glucose concentration of 3.9 mmol/l the patients should be concerned about the possibility of developing hypoglycemia (Cryer, 2005).

People with type 1 diabetes can suffer at least two episodes of symptomatic hypoglycemia and a number of asymptomatic hypoglycemia per week (Cryer, 2005; Cryer, Davis, & Shamoon, 2003). However, hypoglycemia is less frequent in type 2 diabetes and becomes a major problem later in the course of the disease (Cryer, 2008a; Cryer, et al., 2003). Hypoglycemia is a limiting factor in the glycemic management of diabetes (Cryer, 2008a, 2012a). It causes recurrent physical and psychological morbidity and even some mortality in most people with type 1 diabetes and advanced type 2 diabetes who are absolutely insulin-deficient (Cryer, 2012a).

Symptoms of hypoglycemia (Towler, Havlin, Craft, & Cryer, 1993) are categorized as neuroglycopenic (those that are the direct result of brain glucose deprivation per se) and neurogenic (or autonomic), those that are largely the result of the perception of
physiological changes caused by the sympathoadrenal (largely the sympathetic neural) (Towler, et al., 1993) discharge triggered by hypoglycemia. Neuroglycopenic manifestations include cognitive impairments, behavioral changes and psychomotor abnormalities, and, at lower plasma glucose concentrations, seizure and coma. Adrenergic neurogenic symptoms include palpitations, tremor, and anxiety/arousal (Towler, et al., 1993). Cholinergic neurogenic symptoms include sweating, hunger, and paresthesias (Towler, et al., 1993). Central, as well as peripheral, mechanisms may be involved in the generation of some symptoms such as hunger (Cryer, et al., 2003). Awareness of hypoglycemia is largely the result of the perception of neurogenic symptoms (Towler, et al., 1993). Pallor and diaphoresis (the result of adrenergic cutaneous vasoconstriction and cholinergic stimulation of sweat glands, respectively) are common signs of hypoglycemia (Cryer, 2008a). Rarely, hypoglycemia causes sudden, presumably cardiac arrhythmic, death or, if it is prolonged and profound, brain death (Adler et al., 2009; Cryer, 2007). Recent reports indicate that 6% to 10% of deaths of people with type 1 diabetes mellitus are caused by hypoglycemia (Jacobson et al., 2007; Skrivarhaug et al., 2006)

1.1.1 Normal counter-regulatory hormone responses to hypoglycemia

Multiple mechanisms are involved in the normal defense against falling plasma glucose concentrations, but some are more important than others (Cryer, 2008a). Falling plasma glucose concentrations elicit a sequence of responses that normally prevent or rapidly correct hypoglycemia. The physiological defenses against declining plasma
glucose concentrations include 1) a decrease in insulin secretion, 2) an increase in glucagon secretion, and, in absence or deficiency of the latter, an increase in epinephrine secretion (Cryer, 2006). The behavioral defense is the ingestion of carbohydrates prompted by the awareness of hypoglycemia (Cryer, 2008a; DeRosa & Cryer, 2004; Towler, et al., 1993). I will discuss each of these below.

1.1.1.1 Insulin

The first physiological defense against hypoglycemia is a decrease in pancreatic islet β-cell insulin secretion. That occurs as plasma glucose concentrations decline within the physiological range (4.4-4.7 mmol/l) and increases hepatic (and renal) glucose production with virtual cessation of glucose utilization by insulin-sensitive non-neural tissues (Cryer, 2008a). Reduced insulin secretion is believed to be a result of direct effect of hypoglycemia on pancreatic β-cells (Cryer, 1993). Additionally, norepinephrine from pancreatic sympathetic nerves and epinephrine from adrenal medulla have been suggested to play a role in inhibiting insulin secretion from β-cells (Sherck et al., 2001)(Figure 1-1).

1.1.1.2 Glucagon

The second physiological defense is an increase in pancreatic islet α-cell glucagon secretion. That occurs as plasma glucose concentrations fall just below the physiological range (3.6-3.9 mmol/l)(Cryer, 2008a). To increase blood glucose levels, glucagon promotes hepatic glucose output by stimulating glycogenolysis and gluconeogenesis and by decreasing glycogenesis and glycolysis in a concerted fashion via multiple mechanism
Whether \( \alpha \)-cells directly sense and respond to fluctuations in plasma glucose or whether the response is mediated by the autonomic nervous system and/or the paracrine/endocrine effects of secretory products from other islet cell types have been very hotly debated and in fact remain unresolved (Gromada, et al., 2007). While some studies suggest a direct effect of glucose on \( \alpha \)-cell secretory function (Ravier & Rutter, 2005; Vieira, Salehi, & Gylfe, 2007), other studies have suggested that a direct stimulatory effect of low glucose on the \( \alpha \)-cell seems to be of less physiological importance, and in fact, others have postulated that high glucose could paradoxically augment glucagon release (Franklin, Gromada, Gjinovci, Theander, & Wollheim, 2005; Olsen et al., 2005; Salehi, Vieira, & Gylfe, 2006). More recently, more dominant roles for glucagon secretion have been conferred to intra-islet paracrine and autocrine interactions. For example, reduced intra-islet insulin and the co-released zinc from the pancreatic \( \beta \)-cells, have been suggested to promote glucagon secretion (Hope et al., 2004; Ishihara, Maechler, Gjinovci, Herrera, & Wollheim, 2003; Kawamori et al., 2009; Robertson, Zhou, & Slucca, 2011; Slucca, Harmon, Oseid, Bryan, & Robertson, 2010; Zhou et al., 2004; Zhou, Zhang, Harmon, Bryan, & Robertson, 2007; Zhou et al., 2007). This is an underlying concept of the switch-off hypothesis in the induction of glucagon secretion (Hope, et al., 2004). There are other regulators that the fall in their concentrations as part of the switch off hypothesis have been indicated to stimulate the glucagon secretory response from pancreatic \( \alpha \)-cells during hypoglycemia in normal physiological conditions, including \( \gamma \)-amino-butyric acid (GABA) (Rorsman et al., 1989) and somatostatin (Gerich et al., 1974; Unger & Orci, 1977). In addition, central
and autonomic nervous systems also play a role in regulating glucagon secretion (Ahren, 2000; Evans et al., 2004; Marty et al., 2005).

### 1.1.1.3 Epinephrine and Norepinephrine and Glucocorticoids

The third physiological defense, which becomes critical when glucagon secretion is deficient, is an increase in drenomedullary epinephrine secretion. That, too, occurs as plasma glucose concentrations fall just below the physiological range (3.6-3.9mmol/l) and raises plasma glucose concentrations through increasing glucose production and decreasing glucose utilization by the peripheral tissues (Clarke et al., 1979; Cryer, 2006, 2008a; Rizza, Cryer, & Gerich, 1979). Epinephrine response may not be critical in correcting hypoglycemia unless glucagon secretion is deficient, which is the case in type 1 diabetes or advanced type 2 diabetes (Clarke, et al., 1979; S. N. Davis, Fowler, & Costa, 2000; Rizza, et al., 1979).

It has been shown that norepinephrine contributes to increasing blood glucose during hypoglycemia via the same mechanisms as epinephrine (Hansen, Firth, Haymond, Cryer, & Rizza, 1986); however, its concentration in the peripheral blood is much less than epinephrine (Shum et al., 2001)

Glucocorticoids, cortisol in human and corticosterone in rodents, are not part of the primary defense against hypoglycemia (Hoffman, Sinkey, Dopp, & Phillips, 2002). They support glucose production during prolonged hypoglycemia and are normally triggered at the glycemic threshold of 3.2mmol/l (Mitrakou et al., 1991). They stimulate
gluconeogenesis and reduce glucose utilization (De Feo et al., 1989). They also suppress insulin’s inhibitory effect on glucose production (Dirlewanger et al., 2000).

1.2 Hypoglycemia in Type 1 and Type 2 Diabetes mellitus

Because of the effectiveness of physiologic defenses, and in particular in intact glucagon secretory response, hypoglycemia is a distinctly uncommon clinical event except in people with diabetes who use medications, such as a sulfonylurea, or exogenous insulin, that raise circulating insulin concentrations to lower their plasma glucose levels (Cryer, 2012a). In that setting where there is a defective glucagon response (see below), hypoglycemia is common. Indeed, hypoglycemia is the limiting factor in the glycemic management of diabetes (Cryer, 2012a).

Hypoglycemia in diabetes is fundamentally iatrogenic, the result of relative or absolute therapeutic hyperinsulinemia that causes the plasma glucose concentration to decline. However, that alone seldom results in hypoglycemia. Rather, hypoglycemia is typically the result of the interplay of therapeutic hyperinsulinemia and compromised counter-regulatory defenses against the falling plasma glucose concentrations (Cryer, 2012a; Dagogo-Jack, Craft, & Cryer, 1993; Segel, Paramore, & Cryer, 2002). With different time courses, the pathophysiology of glucose counterregulation is the same in type 1 diabetes and advanced (i.e., absolutely endogenous insulin deficient) type 2 diabetes (Cryer, 2008a, 2012a; Dagogo-Jack, et al., 1993; Segel, et al., 2002). Because absolute β-cell failure occurs rapidly in type 1 diabetes but slowly in type 2 diabetes, the
syndromes of defective glucose counterregulation and hypoglycemia unawareness develop early in type 1 diabetes but later in type 2 diabetes (Cryer, 2012a).

1.3 Defective Counter-regulation in Diabetes

The compromised defenses include loss of the decrease in insulin and loss of the increase in glucagon as plasma glucose concentrations fall (Cryer, 2012a; Dagogo-Jack, et al., 1993; Segel, et al., 2002). Some studies also include attenuated adrenomedullary and sympathetic neural responses to falling plasma glucose concentrations resulting in the clinical syndromes of defective glucose counterregulation and hypoglycemia unawareness, respectively, collectively termed hypoglycemia-associated autonomic failure in diabetes (Cryer, 2012a).

1.3.1 Glucagon counter-regulation in diabetes

In type 1 diabetes and advanced type 2 diabetes, the absence of an increment in glucagon secretion, in the setting of an absent decrement in insulin secretion and an attenuated increment in sympathoadrenal activity, in response to falling plasma glucose concentrations plays a key role in the pathogenesis of iatrogenic hypoglycemia (Bolli et al., 1983; Cryer, 2008a, 2012a; Cryer, et al., 2003; Dagogo-Jack, et al., 1993; Rizza, et al., 1979; Segel, et al., 2002). In type 1 diabetes, this defect usually occurs within the first few years of the onset of the disease (Bolli, et al., 1983). This defect may be specific to insulin induced hypoglycemia as the glucagon response to neurogenic stress (Miles, Yamatani, Lickley, & Vranic, 1991; J. T. Y. Yue et al., 2006) and exercise (Purdon et al.,
1993; Sigal et al., 1994) is normal. The exact mechanisms underlying the defect in glucagon response are still unclear. It has been suggested that the glucagon secretion is impaired as the result of loss of the decrement reduction in intra-islet insulin due to loss of β-cell function, which has been postulated to be the underlying basis of loss of insulin switch-off mechanisms in type1 diabetes. Others have suggested the increased α-cell sensitivity to insulin due to absent β-cell function in type 1 diabetes (Taborsky, Ahren, & Havel, 1998) and a defect in autonomic response to hypoglycemia as another contributing underlying mechanisms of dysregulated glucagon secretion (Taborsky, Ahren, Mundinger, Mei, & Havel, 2002). Also, it has been shown that infusion of somatostatin evoked a switch off effect on α-cell during hypoglycemia, which when blocked, can restore glucagon secretion (Farhy et al., 2008). Other studies have suggested the increased pancreatic somatostatin in diabetes as the cause of impaired glucagon secretion in type 1 diabetes (Rastogi, Lickley, Jokay, Efendic, & Vranic, 1990). In addition to local intra-pancreatic networks, there are a number of studies that have suggested that the central nervous system contribute to the perturbation of glucagon secretion (Borg et al., 1999; Paranjape et al., 2010).

### 1.3.2 Epinephrine and norepinephrine and glucocorticoids counter-regulation in diabetes

The epinephrine response to hypoglycemia is critical in a setting of deficient glucagon counterregulation to hypoglycemia (Cryer, 2008a). The individuals lacking both glucagon and epinephrine counterregulation are at 25-fold or greater risk of severe hypoglycemia compared to individuals lacking glucagon but have an intact epinephrine
response (Cryer, 2008a, 2008b, 2012b). Epinephrine counterregulation to hypoglycemia has been shown to be either impaired (Bolli, et al., 1983; Chan et al., 2002; Dagogo-Jack, et al., 1993; K. Inouye et al., 2002) or not impaired (Kinsley & Simonson, 1996) in type-1 diabetes. The epinephrine defect in diabetes could also be stressor specific as it is reduced in response to hypoglycemia and cold stress (Kinsley, Widom, Utzschneider, & Simonson, 1994) but not to exercise (Marliss & Vranic, 2002).

It has been suggested that the prior glycemic control profile of the diabetic individuals contributes to the extent of impairment in the epinephrine response (M. Davis, Mellman, Friedman, Chang, & Shamoon, 1994). In addition, since tight control of blood glucose levels is associated with increased frequency of hypoglycemic episodes, recurrent hypoglycemia may be the possible cause of impaired epinephrine response in type 1 diabetes (K. E. Inouye, Chan, Yue, Matthews, & Vranic, 2005). Many studies have suggested the same central nervous system regulatory mechanisms for epinephrine and glucagon responses particularly in regard to the role of the ventromedial hypothalamus (Chan, Zhu, Ding, McCrimmon, & Sherwin, 2006).

Lastly, in regard to norepinephrine and glucocorticoid responses to hypoglycemia, there are various reports suggesting their decreased (K. Inouye, et al., 2002) or unchanged (Dagogo-Jack, et al., 1993) responses in diabetes. Similar to epinephrine, prior strict glycemic control and the consequent recurrent hypoglycemia may influence the impairment of these responses (Kinsley & Simonson, 1996).
Figure 1-1. Physiology (normal) and pathophysiology (endogenous insulin-deficiency in type 1 diabetes) in glucagon secretory responses to hypoglycemia. Normally, a decrease in plasma glucose causes a decrease in β-cell insulin secretion that signals an increase in α-cell glucagon secretion during hypoglycemia. In the setting of β-cell failure in type 1 diabetes, a decrease in plasma glucose cannot cause a decrease in β-cell insulin secretion, and the absence of that signal results in no increase in pancreatic α-cell glucagon secretion during hypoglycaemia (adapted from (Cryer, 2012a)).
1.4 Somatostatin

1.4.1 Somatostatin functions in pancreatic islets

Somatostatin tightly controls the secretion of glucagon and insulin, two major hormones regulating the glucose homeostasis (Mathias Z. Strowski & Blake, 2008). There are two major circulating SST-isoforms, which consist of 14 and 28 amino acids respectively and are processed from a common precursor protein (Mathias Z. Strowski & Blake, 2008). The primary secretory product of pancreatic D-cells is somatostatin-14 (Patel, Wheatley, & Ning, 1981), which contributes to less than 5% of circulating somatostatin in adult human (Taborsky & Ensinck, 1984). However, the increase in postprandial plasma concentration of somatostatin is mostly due to increased circulating somatostatin-28, which is derived from the intestinal epithelium (Patel & O'Neil, 1988).

The main function of somatostatin in the pancreas is to inhibit the insulin and glucagon secretion (D. J. Koerker et al., 1974). In addition to its potent antisecretory effect, somatostatin also inhibits the gene expression of glucagon and insulin (Moller, Stidsen, Hartmann, & Holst, 2003).

There is much evidence in support of paracrine mechanism of somatostatin-14 action on pancreatic α and β-cells. Firstly, D-cells are in close proximity to α- and β-cells shown in rat and human islets (Orci & Unger, 1975). Secondly, administration of anti-somatostatin antibody to bind to endogenous somatostatin in isolated islets resulted in increased secretion of both insulin and glucagon (Itoh, Mandarino, & Gerich, 1980). Thirdly, immune-neutralization of pancreatic somatostatin in the perfused isolated
pancreata showed similar effects (Brunicardi et al., 1994; Kleinman et al., 1994). Fourthly, the oscillatory pulses of somatostatin and glucagon release are temporally anti-synchronous (Salehi, Qader, Grapengiesser, & Hellman, 2007). Lastly, the use of specific somatostatin receptor agonists (Singh et al., 2007), antagonists (Cejvan, Coy, & Efendic, 2003), somatostatin (Hauge-Evans et al., 2009), and somatostatin receptor knock-out mice (M. Z. Strowski, Parmar, Blake, & Schaeffer, 2000) have collectively and uniformly confirmed the paracrine inhibitory action of pancreatic somatostatin on glucagon and insulin secretion from α- and β-cells.

1.4.2 Somatostatin receptors

Somatostatin induces its effects on the target cells through five different somatostatin receptor subtypes (SSTR1-5).

Rat and human pancreatic islets express all five SSTR subtypes, with each SSTR subtype displaying a distinct distribution on specific islet cells (Mathias Z. Strowski & Blake, 2008). Human pancreatic α and β-cells show a high expression of SSTR2 (Singh, et al., 2007; Mathias Z. Strowski & Blake, 2008). The use of specific SSTR agonists confirmed SSTR2 activity on both α- and β cells in isolated human islets (Singh, et al., 2007). In isolated human pancreatic islets, SSTR2 is the predominant subtype that mediates the suppression of glucagon release by somatostatin (Singh, et al., 2007). Somatostatin inhibition on insulin secretion from β-cell was also shown to be mediated by SSTR2 in isolated perfused human pancreas (Brunicardi et al., 2003; Moldovan et al.,
1995) as well as in isolated pancreatic islets of hamsters (Yao, Gill, Martens, Coy, & Hsu, 2005).

In rodent islets, SSTR2 is the predominant subtype on α-cells while SSTR5 is the predominant SSTR subtype on β-cells (Mitra et al., 1999). This is consistent with subsequent studies showing that SSTR2 mediated suppression of glucagon secretion from α-cells (Cejvan, et al., 2003; M. Z. Strowski, et al., 2000), and that SSTR5 mediated inhibition of insulin release from pancreatic β-cells (Kailey et al., 2012; Mitra, et al., 1999; Rossowski & Coy, 1994; M. Z. Strowski et al., 2003; M. Z. Strowski, et al., 2000).

### 1.4.3 Somatostatin in diabetes

The number of D-cells has been reported to be increased in patients with type 1 diabetes (Orci et al., 1976; Rahier, Goebbels, & Henquin, 1983). These patients also have an elevated level of basal plasma somatostatin (Segers, De Vroede, Michotte, & Somers, 1989) and pancreatic somatostatin content (Orci, et al., 1976). There are numerous studies demonstrating increased pancreatic and plasma somatostatin levels as well as the number of pancreatic D-cells in models of type-1 diabetes, including streptozotocin (STZ) diabetic rats, Non obese diabetic (NOD) mice and alloxan diabetic dogs (Mancera, Gomez, & Pisanty, 1995; Orci, et al., 1976; Papachristou, Pham, Zingg, & Patel, 1989; Patel & Weir, 1976; Rastogi, et al., 1990; Shi et al., 1996). In diabetic bio-breeding diabetic prone (BBDP) rats, the model we have used in this thesis, the plasma level of somatostatin was reported to be increased; however the pancreatic content of
somatostatin was reported to be reduced (Patel, Ruggere, Malaise-Lagae, & Orci, 1983).

There is evidence that insulin treatment can reduce and/or partially normalize the elevated plasma somatostatin in diabetic animal models (Papachristou, et al., 1989; Patel, et al., 1983; Rastogi, et al., 1990) and in type 1 diabetic patients (Segers, et al., 1989).

1.4.4 Somatostatin receptor type 2 antagonist PRL-2903

Various antagonists with different pharmacological selectivity for particular SSTRs have been synthesized. Of these, the SSTR2 antagonist peptide used in the present thesis is PRL-2903, also known as DC-41-33 and BIM-234548 (Hocart, Jain, Murphy, Taylor, & Coy, 1999). This octapeptide, synthesized by Dr. David Coy at Tulane University (New Orleans, LA, USA) (Hocart, et al., 1999), has a molecular weight of 1160 da, PRL-2903 is selective for SSTR2 over SSTR3 and SSTR5 by 10 and 40 fold, respectively, and have negligible binding affinity to SSTR1 and SSTR4 (Hocart, et al., 1999).

1.5 Animal models of type 1 diabetes: BBDP diabetic rats Vs Streptozotocin induced diabetic rats

Type 1 diabetes comprises ~10% of all patients with diabetes mellitus, and its prevalence is increasing. Affected individuals require lifelong injections of insulin for survival (Green & Patterson, 2001). The disease results from inflammatory infiltration of
the islets of Langerhans (insulitis) and selective destruction of insulin-producing beta cells (Atkinson & Eisenbarth, 2001).

There are different rodent models to study type 1 diabetes. Two major models are streptozotocin (STZ) diabetic rats and Biobreeding (BB) diabetic rats. In the STZ model, STZ, an antibiotic, induces diabetes by chemical destruction of β-cells. The BB model is considered to be an autoimmune type 1 diabetic model, thus more closely mimic the human type-1 diabetes. I will discuss each of these two models.

1.5.1 BBDP diabetic rats

BB rats are the most extensively studied rat model of type-1 diabetes. They are derived from a Canadian colony of outbred Wistar rats developed in the 1970s, which exhibited spontaneous hyperglycemia and ketoacidosis (Nakhooda, Like, Chappel, Murray, & Marliss, 1977). These original colonies were the founders for two subsequent colonies that were later used to establish all other BB rat colonies. One colony was established in Worcester, Massachusetts (BB/Wor), and were inbred, and spontaneously diabetic, and this colony was formally designated as Worcester diabetes-prone BB rats (BBDP/Wor) (BIOMERE LLC, Worcester, MA, USA) (Mordes, Bortell, Blankenhorn, Rossini, & Greiner, 2004). This colony became the commercial source used by many investigators, including ourselves in this thesis.

BBDP/Wor rats of both sexes develop pancreatic insulitis that is rapidly followed by selective destruction of beta cells and frank diabetes between 50 and 90 days of age (Stubbs, Guberski, & Like, 1994). It is noteworthy that the natural course of insulitis in
the spontaneously diabetic BB rat is different from other models of autoimmune diabetes such as the non-obese diabetic (NOD) mouse, the most commonly used mouse model of type-1 diabetes. In the BBDP rat, there is no significant or persistent infiltration in the regions adjacent to the islet (“peri-insulitis”) before progression to frank insulitis and overt diabetes, which is observed in NOD mouse islets. Insulitis in BB rats is morphologically similar to that observed in human type 1 diabetes and features a predominance of Th1-type lymphocytes (Kolb et al., 1996). After the onset of hyperglycemia, residual “end-stage” islets are small, distorted, and composed predominantly of non β-cells. Unless treated with exogenous insulin, hyperglycemic BB rats quickly progress to diabetic ketoacidosis that is fatal (Mordes, et al., 2004).

In the current study, we have employed the diabetic BBDP/Wor rats as the model of autoimmune diabetes because it etiologically more closely mimics the human autoimmune diabetes, and because we can track the course of the disease in established type 1 diabetes. Towards the latter, we wanted the type-1 diabetic model to also mimic the clinical treatment course of the human disease, that is to be dependent and to receive exogenous insulin as treatment. This also mimics type 1 diabetic patients who have to insulin treatments. The STZ-induced diabetic rat model, develop diabetes as the result of a chemical destruction of the insulin secreting β-cells, which is completely different from the disease process in human type 1 diabetes.

1.5.2 STZ induced diabetic rats
Streptozotocin, an antibiotic derived from Streptomyces achromogenes, is structurally a glucosamine derivative of nitrosourea. The types of diabetes that is induced and other characteristics of beta cell damage differ depending on the dose and frequency of the STZ administered and also the different animal species used. Single diabetogenic dose of STZ (70-250mg/kg, body weight) has been demonstrated to induce complete destruction of beta cells in most species within 24 hour. STZ induces diabetes in almost all species (Junod et al., 1967). Diabetes can be induced by STZ either by single injection of STZ or by multiple low dose injections of STZ. STZ is the most commonly used drug for induction of diabetes in rats. Initial hyperglycemia is observed at one hour after the STZ injection followed by hypoglycemia; and then a hyperglycemic state observed at 48 hours which peaks at 48-72 hours; this hyperglycemic state is maintained thereafter (Bonner-Weir, Trent, Honey, & Weir, 1981).

1.6 Islet cell composition, normal vs. type 1 diabetes

The islets of Langerhans are located in the pancreas. The primary cell types within the rodent islet are alpha-, beta-, and delta-cells which contribute approximately 15-20%, 60-80%, and 5-10%, respectively (Unger & Orci, 1981). In human, the relative percentages are 40%, 50% and 10%, respectively (Cabrera et al., 2006). In a normal rodent islet, alpha-cells form a thin layer of cells on the islet surface that enwraps the large population of beta-cells which are clustered within the islet core. In between alpha- and beta-cells interspersed the delta-cells (Unger & Orci, 1976). This fine anatomical subdivision of rodent islets allows immediate contact of alpha-cells with
other islet cell types, and is particularly crucial for the secretion, regulation, and intercellular communication of alpha-cells with other islet cell types. At present, the underlying mechanisms of these intraislet paracrine interactions remain largely unclear (Unger & Orci, 1976).

This cellular arrangement within rodent islets does not exist within the human islets. Alpha-, beta-, and delta-cells are randomly scattered throughout the human islets and are frequently found aligning along the blood vessels (Cabrera, et al., 2006). However, despite this difference, alpha-cells remain largely in contact with adjacent delta- and beta-cells, which, similar to rodent alpha-cells, would have similarly important functional implications on paracrine regulation.

Studies on STZ diabetic rats showed a highly significant decrease in the number and volume of insulin immunofluorescent cells per pancreas indicating beta cell destruction; and an increase in the total number and volume of somatostatin immunofluorescent delta cells per pancreas. But the number and volume of glucagon immunofluorescent alpha cells per pancreas did not differ significantly from control rats despite the apparent increases per diabetic islet. They interpreted these findings as evidence of D-cell hypertrophy and hyperplasia in diabetes (Orci, et al., 1976).

Studies on BB diabetic rats showed the virtual elimination of betacells and severe reduction of deltapcells early in diabetes. The alphacells were preserved initially, but eventually they, too, underwent attrition. Thus, the "terminal" islets from chronic diabetic insulin-treated rats, exhibit 85 to 100% loss of all islet cells. Such a generalized involvement of all islet cells is probably caused by the islet inflammatory process and is a
feature of other forms of diabetes also characterized by insulitis, for example virus-induced diabetes in mice (Patel, et al., 1983).

In a study of two diabetic human pancreata, islets were clearly less numerous than in the normal pancreases. No insulin immunofluorescent cells could be identified in the diabetic islets, which consisted largely of glucagon-immunofluorescent cells; somatostatin-immunofluorescent cells were also abundant. Morphometric analyses revealed both cell types to be significantly increased above the control values (Orci, et al., 1976).

1.7 Rationale

Hypoglycemia has been well recognized to be one of the most serious acute complications of type-1 diabetes; thus a major limiting factor in intensive insulin treatment targeted at tight control of blood glucose (Cryer, 2008a). The main objective of this thesis is to determine a means to improve or prevent hypoglycemia in type-1 diabetes.

In type-1 diabetes, hypoglycemia counter-regulation is impaired, the major component being the greatly diminished glucagon secretory response to hypoglycemia in type =1- diabetes (Cryer, 2008a, 2008b). The precise mechanisms underlying the impaired glucagon secretory response in type-1 diabetes during hypoglycemia are yet to be elucidated (Cryer, 2012a). There is considerable evidence that accentuated inhibitory effect of excessive pancreatic somatostatin on glucagon release in the absence of the tonic inhibitory effect of insulin compromise the impairment of glucagon counter-
First, it has been suggested that somatostatin released from islet D-cells tightly controls glucagon release through a local paracrine action (Hauge-Evans, et al., 2009; Mathias Z. Strowski & Blake, 2008; Unger & Orci, 1977). Second, the number of D-cells is increased in type-1 diabetic humans and rodents (Mathias Z. Strowski & Blake, 2008). Third, it has been shown that plasma somatostatin and the pancreatic prosomatostatin mRNA and somatostatin protein levels are increased in diabetic humans (Orci, et al., 1976), dogs (Rastogi, et al., 1990), and rodents (Patel, et al., 1983; J. T. Yue, et al., 2012). Fourth, exogenously administered somatostatin (SST) inhibits the release of glucagon (Donna J. Koerker et al., 1974) from the endocrine pancreas. Fifth, administration of a somatostatin receptor antagonist increases the glucagon secretory response to hypoglycemia in STZ-diabetic rats (J. T. Yue, et al., 2012); and lastly, the stimulated glucagon secretion was increased in SSTR2 knockout mice (M. Z. Strowski, et al., 2000).

As mentioned above, it is well established that somatostatin induces its inhibitory effect on glucagon secretion from α-cells through SSTR2 and this finding has been supported by studies using the specific SSTR2 agonist (Singh, et al., 2007) and antagonist (Cejvan, et al., 2003) as well as SSTR2 knockout mice (M. Z. Strowski, et al., 2000). The use of specific SSTR2 antagonist, PRL-2903 (Hocart, et al., 1999) in isolated perfused pancreatic islets and isolated perfused pancreata of non-diabetic rats has confirmed that SST can act as an intra-islet regulator of glucagon secretion in rats,
thereby antagonizing the SSTR2, which would explain the enhancement of e arginine-stimulated glucagon secretion (Cejvan, et al., 2003).

We had previously hypothesized that antagonising the somatostatin action by a specific SSTR2 antagonist can restore the impaired hypoglycemia countergulation in type-1 diabetes (J. T. Yue, et al., 2012). However, in that previous study employing the STZ-induced diabetic rat model, insulin treatment was not required, thus not truly mimicking the human disease. Nonetheless, antagonising the SSTR2 by PRL-2903 could normalize the glucagon and corticosterone counter-regulatory responses to insulin-induced hypoglycemia in the STZ-treated rat model (J. T. Yue, et al., 2012).

The effect of SSTR2 antagonism has been never tested in the autoimmune type-1 BB rat model which resembles human type 1 diabetes in many aspects. Furthermore, it would be of particular interest to explore the effect of SSTR2 antagonism on restoring the counter-regulatory hormones secretory responses to hypoglycemia in diabetic BB rats that receive exogenous insulin treatment using slow releasing subcutaneous insulin pellets which clinically mimics the insulin therapy in diabetic patients.

0To investigate the specific and direct effects of SSTR2 blockade on the pancreatic alpha-cells to cause increase in glucagon secretion independent of any confounding systemic factors, we have employed the thin pancreatic slices perifusion method. Previously, we had used the thin pancreatic slices to study the in-situ electrophysiology of the particular ion channels in mouse pancreatic α- and β-cells (Huang, Rupnik, & Gaisano, 2011; Huang et al., 2012). This is the first time that the thin fresh pancreatic slices have been used in a perifusion setting to explore the real-time hormonal secretion
from diabetic rat and normal human pancreata. By using this approach we have surmounted the technical limitations of the conventional methods being used to study the pancreatic alpha cells (Huang, et al., 2011; Speier & Rupnik, 2003), particularly in diabetic pancreatic islets. Since the islets are extremely damaged in diabetes, conventional methods of islet isolation and dispersion into single cells are not reliable; and this in part explains why previous reports have been inconsistent with regards to the functions and cellular interactions of islet cells (Speier & Rupnik, 2003; Speier, Yang, Sroka, Rose, & Rupnik, 2005). The focus of my thesis study is to investigate the paracrine interaction of D-cellsomatostatin and alpha-cell glucagon secretory response in a type-1 BB rat model. This assay of pancreas slice perfusion would be a more ideal method to study the pancreas without interference of other factors like the central and autonomic nervous systems present in the in-vivo settings or in the ex-vivo isolated whole-pancreas perfusion assay. Furthermore, by deploying this technique to human pancreas, we have been able to study the normal human pancreatic islets also in situ in their normal architecture by using a very small fresh pancreatic specimens obtained from surgical resection of pancreatic cancer. The latter would provide initial insight as to whether these findings of SSTR2 blockage in enhancing glucagon secretion during hypoglycemia can be translatable to humans.

1.8 Hypothesis

We hypothesise that the impaired glucagon counter-regulation in type 1 diabetes is due to the accentuated somatostatin inhibitory effect on α-cells in the
absence of endogenous insulin; thus antagonising the somatostatin receptor type 2, in order to block the somatostatin inhibitory effect on α-cells would improve the compromised glucagon counter-regulatory response to hypoglycemia in autoimmune type 1 diabetic BBDP rats. (Figure 1-4)

**Figure 1-4. Alpha, beta and delta cell interactions during hypoglycemia.** In absence of the tonic effect of insulin, somatostatin is the only endogenous inhibitor of glucagon release that exerts a strong inhibitory effect on α-cell. Therefore, when an antagonist blocks the somatostatin receptors on α-cell, despite the inhibitory effect of the injected insulin, α-cell can release normal amount of glucagon during hypoglycemia (Gaisano, et al., 2012; Vranic, 2010).
1.9 Objectives

1. To investigate in the autoimmune type-1diabetic model - diabetic BB rats the in vivo effects of SSTR2 antagonism on restoring counter-regulatory glucagon secretory responses by employing hyper-insulinemic hypoglycemic clamps.

2. To explore the catecholamines (epinephrine and norepinephrin) and corticosterone responses to hypoglycemia in type 1 diabetic BBDP rats and whether these responses will be improved by antagonising the SSTR2 during the hyper-insulinemic hypoglycemic clamp.

3. To unequivocally demonstrate and confirm the specific action of SSTR2 blockade on increasing glucagon secretion by employing the pancreatic slices perifusion method in diabetic, non-diabetic BB rats.

4. To deploy the pancreatic slices perifusion technique to normal human pancreas in order to demonstrate the specific action of SSTR2 blockade on increasing glucagon secretion.
Chapter 2 Research design and methods

2.1 In-vivo Studies

2.1.1 Animals

All procedures were in accordance with Canadian Council on Animal Care Standards and were approved by the University of Toronto Animal Care Committee.

Male diabetic prone BB/Wor rats (BIOMERE LLC, Worcester, MA, USA) were housed in a sterile animal facility, fed a standard pellet diet, and maintained on a 12-h/12-h day/night cycle. The age-matched BBDP rats which did not become diabetic at age range of 60-150 days, were used as controls. Diabetic rats were treated by subcutaneous implantation of 1.5-2 insulin pellets (Linplants; LinShin Canada, Scarborough, Toronto, ON, Canada) once they become diabetic (random BS> 22mmol/l on two sequential measurements). The implants released insulin at ∼2 units/24 h.

2.1.2 Somatostatin receptor type2 antagonist (PRL-2930) solution

SSTR2a (PRL-2930) was generously provided by Dr. David H. Coy (Tulane University, New Orleans, LA) (Hocart, et al., 1999). The SSTR2a solution was prepared by dissolving a calculated dose of the antagonist in 1% acetic acid and 0.9% saline.
2.1.3 Surgical procedures and hypoglycemic clamp experiments

Animals were studied in 4 different groups: Diabetic vehicle (n=8), Diabetic+SSTR2a (n=8), Non-Diabetic vehicle (n=5) and Non-Diabetic+SSTR2a (n=6).

The Surgery and the clamp procedures were performed in accordance with our previous study in STZ-treated diabetic rats (J. T. Yue, et al., 2012). Surgery was performed 7 days prior to the hypoglycaemic clamp experiments. In case of BBDP diabetic rats, this was 14 days after the insulin pellet implantation. Under general anaesthesia, by isoflurane inhalation, the left carotid artery and right jugular vein were catheterized for blood sampling and infusion of test substances, respectively.

Immediately after the clamp experiments, the rats were sacrificed and the whole pancreata were dissected, frozen on dry ice and transferred to -80°C for latter determinations of total pancreatic content of somatostatin and glucagon. The whole pancreata of the age-matched BBDP Diabetic (± insulin pellet implantation) and non-diabetic rats which had not undergone hypoglycemic clamp study were also dissected and preserved.

On the day of the clamp experiment, the rats were weighed, connected to infusion catheters and acclimatized for 1 hour. Any stress trigger was strictly avoided during this period and throughout the whole hypoglycemic clamp procedure. After obtaining a baseline blood sample at t = -60 min, the rats underwent a 3-hour infusion of vehicle (1% acetic acid in 0.9% saline) or SSTR2a (1500 nmol/kg/h) at an infusion rate of 1ml/h with a digital syringe infusion pump (Harvard Apparatus, Holliston, MA, USA). In order to induce hypoglycaemia to a target level of 3±0.5mmol/l, insulin infusion at the constant
rate of 20-50 mU/kg/min together with variable rates of glucose infusion were started at $t = 0$ min.

During the clamp, blood samples were obtained from the carotid catheter every 10 minutes in capillary tubes coated with Kalium-EDTA (Microvette CB 3000, Sarstedt Inc., Montreal, QC Canada) and immediately centrifuged in room temperature in order to measure the plasma glucose levels using a glucose analyzer (Analox Glucose analyzer, Analox instrument, London, UK).

**Figure 2-1**

**In vivo Studies**

- Diabetes
- Insulin pellet implantation
- Surgery
- Hypoglycemic clamp

**Hyperinsulinemic hypoglycemic clamp**

- Catheters opened
- Insulin infusion (20-50 mU/kg/min) + Variable glucose
2.1.4 Plasma hormone measurements

Blood samples were obtained at t = -60 min, then every 30 minutes from t=0 min in ice-chilled tubes containing 5µl of 100mM EDTA (Ethylenediamine tetraacetic acid) solution and 30 KIU aprotinin (APR600, BioShop Canada Inc., Burlington, ON, Canada); followed by centrifugation at 12000 rpm at 4°C to separate the plasma. The plasma was aliquoted in multiple tubes and immediately frozen on dry ice and transferred to -80°C awaiting measurements of glucagon by radioimmunoassay (rat glucagon RIA kit, EMD Millipore, Darmstadt, Germany), and corticosterone, nor/epinephrine by enzyme-linked immunosorbent assays (corticosterone ELISA kits by ALPCO Diagnostics, Salem, NH, USA; nor/epinephrine by 2-Cat Plasma ELISA kit, Labor Diagnostica Nord GmbH & Co. KG, Nordhorn, Germany), and somatostatin by enzyme immunoassay (Extraction free, rat somatostatin-14 EIA kit, Bachem Group, Bubendorf, Switzerland).

2.1.5 Total pancreatic glucagon and somatostatin protein content

The frozen harvested pancreata were placed in acid-ethanol mixture (1.5%HCl in 70% EtOH), incubated twice overnight at -20°C. Between the two incubation periods, the pancreata were homogenized; following centrifugation, and neutralization by 1M pH 7.5 Tris buffer, somatostatin and glucagon levels were determined by RIA and EIA, respectively. The derived hormone concentrations were then normalized to the total
pancreatic protein content determined by modified Lowry method (Markwell, Haas, Bieber, & Tolbert, 1978).

2.2 In-vitro studies

2.2.1 Animals

Male diabetic prone BB/Wor rats (BIOMERE LLC, Worcester, MA, USA) (N=6) were used for the in-vitro studies. The age-matched BBDP rats which did not become diabetic at age range of 60 to 150 days, were used as controls (N=6). The insulin treatment was as previously mentioned in the in-vivo section.

2.2.2 Human pancreas specimens

All procedures which involved human pancreas tissue were approved by the University Health Network Research Ethics Board (Protocol #10-0393-T) and written consents were obtained from the source patients.

The human pancreas specimens were obtained from the normal margins of the resected pancreata of the pancreatic cancer patients who had been operated at Toronto General Hospital. Immediately after the resection, the specimens were sent to the surgical pathology laboratory to be evaluated, and a normal portion not required for pathology review, kept in extra cellular solution prior to the experiments.

2.2.3 Pancreatic slices preparation
Pancreatic slices were prepared as previously described (Huang, et al., 2011; Huang, et al., 2012). After sacrificing the rat, the rat abdominal cavity was immediately opened, and the common bile duct clamped. 3 ml of 37°C low melting 1.9% agarose gel (15517-022, Invitrogen, Camarillo, CA) was injected into the pancreas via the proximal end of the common bile duct using a PE-50 tube. The injected pancreas was resected and cut into smaller pieces which were then embedded in 1.9% agarose gel and cooled down to become solidified (Figure 2-1). The tissue blocks were placed in Carbogen (5% CO2, 95% O2) bubbled ice-cold extracellular solution and were sliced into 4×4 mm, 140 μm-thick slices at a blade frequency of 70 Hz by a vibrating blade microtome (Vibratome, Leica Microsystems, Mannheim, Germany)(Figure 2-2 A&B). The extracellular solution bathing the pancreas slices was composed of (in mmol/l) 125 NaCl, 2.5 KCl, 1 MgCl2, 2 CaCl2, 1.25 NaH2PO4, 26 NaHCO3, 2 Na pyruvate, 0.25 ascorbic acid, 3 myo-inositol, 6 lactic acid, 7 glucose.

The human pancreas slices were cut out of the agarose gel embedded tissue blocks using the same procedures as mentioned above.

**Figure 2-2**

*Figure 2-2. Pancreatic tissue portions embedded in 1.9% agarose gel and cooled down to become solidified. Courtesy of Dr.Ya-chi Huang 2012*
Figure 2-3. The tissue blocks were placed in Carbogen (5% CO2, 95% O2) bubbled ice-cold extracellular solution and were sliced into 4×4 mm, 140 μm-thick slices at a blade frequency of 70 Hz by a vibrating blade microtome (Vibratome, Leica Microsystems, Mannheim, Germany). Courtesy of Dr. Ya-chi Huang 2012.

Figure 2-4. Perifusion pump and chambers. The pancreatic slices were loaded into the perifusion chambers at 37°C and were perifused by different solutions. The perfusate was collected in ice chilled tubes containing aprotinin.
2.2.4 Pancreas slices perifusion studies

HEPES balanced KRBB solution (KRHB) containing in mmol/l: 135 NaCl, 3.6 KCl, 0.5 NaH2PO4, 0.5 MgCl2, 1.5 CaCl2, 2 NaHCO3, 10 HEPES, 1 or 7 glucose (as indicated in the perifusion protocol) and 1 g/L BSA was used as the perifusion solution with the flow rate of 1 ml/min. Arginine and SSTR2a (PRL-2903) at concentrations of 20 mmol/l and 30 µmol/ml, respectively, were added to the perifusion solution as indicated in the study protocols.

Rat pancreas slice perifusion studies were carried out in the following 4 groups: Diabetic, Diabetic+SSTR2a, Non-Diabetic, Non-Diabetic+SSTR2a. 10 pancreatic slices were loaded into each perifusion chamber (Figure 2-3); the perifusion protocol was initiated with a 20-min equilibration period with KRHB containing 7 mmol/l glucose which was followed by KRHB containing 1 mmol/l glucose (30 minutes), 7 mmol/l glucose (10 minutes) and 1 mmol/l glucose plus 20 mmol/l arginine (30 minutes), respectively. For the Non-diabetic and Diabetic groups treated with SSTR2a, the slices were perifused with SSTR2a at a concentration of 30 µmol/ml for the whole duration of the perifusion procedure (including the equilibration period).

The human pancreas slices were perifused by loading 15-20 slices per chamber. The perifusion protocol was as follows: perifusion of KRHB containing 7 mmol/l glucose (15 minutes as the equilibration period) followed by 1 mmol/l glucose (30 minutes), 1 mmol/l glucose + 30 µmol/ml SSTR2a (30 minutes) and finally a KRHB containing 1 mmol/l glucose and 20 mmol/l arginine (20 minutes).
The samples were collected at 1-min intervals in ice chilled tubes containing 1000KIU aprotinin; the tubes were frozen and stored at -80°C awaiting determination of glucagon levels by RIA. At the end of the experiments, the slices were collected from each chamber and were preserved in acid-ethanol mixture (1.5% HCl in 70% ethanol) and kept at -80°C for the purpose of determining the total glucagon content of the slices.

**Figure 2-5**

**Diabetic or non diabetic BBDP rats pancreatic slice perifusion studies**

![Diagram](image)

**Figure 2-6**

**Normal human pancreatic slices perifusion studies**

![Diagram](image)
3.2.5 Pancreatic slices perifusion secretory glucagon and insulin measurements

The secretory glucagon and insulin levels from the perifusion samples were measured using radioimmunoassay (rat glucagon and insulin RIA kit, EMD Millipore, Darmstadt, Germany). The total glucagon and insulin content of the slices were measured by Acid ethanol (1.5%HCl in 70%EtOH) extraction method as previously mentioned in the in-vivo section of the methods.

2.3 Statistical analysis

Data are presented as mean ± SEM for a given number of observations. Groups of data were compared using ANOVA with Tukey post-hoc test or two tailed paired/unpaired t test (Graphpad, PRISM software, USA), with significance being accepted if P < 0.05.
Chapter 3 Results

3.1 In-vivo studies

3.1.1 Plasma glucose levels during the hyperinsulinemic hypoglycemic clamp experiments

The animals received 1500 nmol/kg/hr SSTR2a or Vehicle solution IV from the beginning of the clamp experiments. Insulin infusion at the rate of 20-50 mU/kg/min along with 50% glucose solution infusion at variable rates were started at t=0. All of the groups reached the target hypoglycemic level (3±0.5 mmol/l) 70 minutes after the start of the insulin infusion and remained at the same level until the end of the experiment. (Figure 3-1)

3.1.2 Glucose infusion rates during the hyperinsulinemic hypoglycemic clamp experiments

The Non-Diabetic SSTR2a group, Non Diabetic Vehicle group and the Diabetic SSTR2a group required very low to no glucose to become and remain hypoglycemic at the target level of 3±0.5 mmol/l. In contrast, the Diabetic Vehicle group had very high glucose requirements in order to maintain the hypoglycemia at the target level of 3±0.5 mmol/l. (Figure 3-2)

3.1.3 Insulin infusion rates during hyperinsulinemic hypoglycemic clamp experiments
The Diabetic Vehicle, Diabetic SSTR2a and the Non Diabetic Vehicle groups received 20 or 30 mU/Kg/min insulin infusion via their jugular vein in order to induce and maintain the target hypoglycemic level during the clamp experiments. The mean ± SEM infusion rates were not significantly different between the Diabetic Vehicle (25±1.89), Diabetic SSTR2a (25.56±1.75) and Non Diabetic Vehicle (26±2.44) groups; however we had to infuse 50 mU/Kg/min for the animals in the Non Diabetic SSTR2a group in order to induce the target hypoglycemic level. These results would suggest that the non-diabetic BB rats receiving SSTR2a treatment became insulin resistant. (Figure 3-3)

### 3.1.4 Plasma hormones levels during hyperinsulinemic hypoglycemic clamp experiments

Glucagon secretion during hypoglycemia was much blunted in Diabetic Vehicle group compared to the Non Diabetic Vehicle group (p<0.0001). Glucagon secretion during hypoglycemia was increased in Diabetic SSTR2a group by almost 2.3 fold compared to Diabetic Vehicle group (p=0.005), and this increase showed full restoration to the same level as the Non Diabetic Vehicle group (p=0.94). SSTR2a treatment also enhanced the glucagon secretion in Non Diabetic SSTR2a group by nearly 3.3 fold compared to Non Diabetic Vehicle group, Diabetic SSTR2a and Diabetic Vehicle groups(p<0.0001). (Figure 3-4 A & B)

Corticosterone secretion in Diabetic Vehicle was not blunted and in fact increased during hypoglycemia, compared to Non Diabetic Vehicle group (p=0.009). SSTR2a treatment in Diabetic SSTR2a group resulted in enhancement of corticosterone secretion
compared to Diabetic Vehicle (p=0.019), Non Diabetic Vehicle (p=0.0041) and Non Diabetic SSTR2a (p=0.006). Corticosterone secretion remained unchanged in Non Diabetic SSTR2a group compared to Non-Diabetic Vehicle group (p=0.412). (Figure 3-5. A & B)

Epinephrine secretion was unchanged by the SSTR2a treatment in Diabetic (p=0.975) and Non Diabetic (p=0.249) groups. Also, the epinephrine response was not compromised in Diabetic vehicle compared to Non Diabetic Vehicle (p=0.412). (Figure 3-6 A & B)

Norepinephrine secretion remained unchanged by the SSTR2a treatment in Diabetic (p=0.626) and Non Diabetic (p=0.059) groups. Diabetes did not affect the norepinephrine response to hypoglycemia. (Figure 3-7 A & B)

Plasma Somatostatin AUC calculations demonstrated that SSTR2a treatment increased the plasma somatostatin during hypoglycemia in Non Diabetic SSTR2a group by almost 3 fold compared to Non Diabetic Vehicle, Diabetic Vehicle and Diabetic SSTR2a groups (p<0.0001). Plasma somatostatin remained unchanged during hypoglycemia in Diabetic SSTR2a group compared to Diabetic Vehicle (p=0.735) and also in Diabetic Vehicle group compared to Non Diabetic Vehicle (p=0.173). (Figure 3-8 A &B)

3.1.5 The whole pancreatic glucagon and somatostatin protein levels immediately following the hyperinsulinemic hypoglycemic clamp experiments
The whole pancreatic glucagon protein content was significantly lower in the Diabetic SSTR2a group immediately after the hypoglycemic clamp compared to Diabetic Vehicle group (p=0.0009), suggesting that the α-cells could secrete more glucagon in response to hypoglycemia in SSTR2a-treated diabetic rats compared to the Diabetic Vehicle group. There was no significant difference between the glucagon protein content of the pancreata between the Diabetic Vehicle, Non-Diabetic Vehicle and Non-Diabetic SSTR2a groups following hypoglycemia, confirming that while there might be enough synthesis of glucagon in α-cells in diabetic rats treated by insulin (Diabetic Vehicle group), the glucagon response to hypoglycemia still remains blunted. (Figure 3-9)

The pancreatic somatostatin content following hypoglycemia was slightly lower in Diabetic SSTR2a group compared to Diabetic Vehicle; however, the difference was not significant (p=0.057). SSTR2a treatment elevated the total pancreatic somatostatin content in the Non Diabetic SSTR2a group compared to Non Diabetic Vehicle; however, the difference was not significant (p=0.055). Diabetic Vehicle and Diabetic SSTR2a had lower pancreatic somatostatin contents compared to Non Diabetic SSTR2a group (p=0.002 and 0.0003 respectively). Also, while the pancreatic somatostatin content was not different between the Diabetic Vehicle and the Non Diabetic Vehicle (p=0.102), SSTR2a treatment significantly decreased the pancreatic somatostatin content following hypoglycemia in Diabetic SSTR2a group compared to Non Diabetic Vehicle (p=0.004). Overall it can be postulated that hypoglycemia results in more somatostatin-14 synthesis in D-cells of the non-diabetic BB rats. (Figure 3-10)
3.1.6 The whole pancreatic glucagon and somatostatin protein levels in the diabetic and non diabetic BB rats not undergone the clamp

The insulin-treated diabetic BBDP rats had a significantly lower pancreatic glucagon protein content compared to untreated diabetic and non diabetic BB rats (p=0.005 and 0.003 respectively), suggesting that the α-cells become more primed to secrete glucagon in insulin-treated diabetic rats. There was no significant difference between the pancreatic glucagon content of untreated diabetic rats and the non diabetic rats (p=0.827). (Figure 3-11)

The insulin-treated diabetic BBDP rats had also a significantly lower pancreatic somatostatin protein content compared to untreated diabetic and non diabetic BB rats (p=0.0006 and 0.004 respectively), suggesting that insulin treatment could correct the high levels of pancreatic somatostatin observed in the untreated diabetic BB rats to some extent. Although the pancreatic somatostatin content was slightly higher in untreated diabetic rats compared to the non diabetic rats, the difference was not statistically significant (p=0.277). (Figure 3-12)

3.2 In-vitro studies

3.2.1 Diabetic BBDP and non diabetic BBDP rat pancreatic slices glucagon secretion in perifusion studies

We next assessed the direct effects of SSTR2a on a-cell glucagon secretion without any confounding systemic factors. We thus employed the pancreatic slice technique
SSTR2a treatment significantly increased glucagon secretion from the diabetic BBDP pancreatic slices in hypoglycemic and basal conditions by approximately 2 fold compared to the control (p=0.015 and 0.047 respectively). In addition, SSTR2a treatment significantly enhanced the arginine-stimulated glucagon secretion from the diabetic BBDP pancreatic slices compared to control diabetic BBDP rats (p=0.003). (Figure 3-13 A & B)

SSTR2a treatment also significantly increased the glucagon secretion from the non-diabetic BBDP pancreatic slices in hypoglycemic and basal conditions by approximately 2.8 fold compared to the control (P=0.001 and 0.009 respectively). In addition, SSTR2a treatment significantly enhanced the arginine stimulated glucagon secretion from the non-diabetic BBDP pancreatic slices compared to the control non-diabetic BBDP rats (p=0.015)(Figure 3-14 A & B.)

3.2.2 Human Pancreatic Slices Glucagon and Insulin Secretion in Perifusion Studies

We next examined whether these observations made on the rat model are translatable to the human clinical situation, employing human pancreas slices which were prepared from normal portions of pancreatic cancer resections. The glucagon secretion from the human pancreatic slices after a short transition showed an obvious enhancement in response to low glucose when SSTR2a was added to the perifusion solution. SSTR2a also enhanced arginine-stimulated release in the low glucose (1mmol/l) condition, which was observed as a prominent peak. AUC calculations of these data from perifusion
assay on human pancreas slices confirmed the enhanced glucagon secretory responses to hypoglycemia by the SSTR2a treatment (Figure 3-15 A & B).

We also measured the simultaneous insulin secretion from the human pancreatic slices during hypoglycemia, as well as when treated with SSTR2a or when arginine. Shortly after adding SSTR2a to the solution, insulin secretion from the slices was increased. Adding arginine resulted in a sharp increase in the insulin secretion from the slices (Figure 3-15 C). This served as a positive control. However, since we had only two replicates of these experiments, we were not able to perform any statistical analysis. The human samples were scarce, and I intend to obtain at least two more human samples.
Figure 3-1. Plasma glucose levels during hyperinsulinemic hypoglycemic clamps experiments. The animals have received 1500 nmol/kg/hr SSTR2a or Vehicle solution IV from the beginning of the clamp experiments. Insulin infusion at the rate of 20-50 mU/kg/min along with 50% glucose solution infusion at variable rates were started at t=0. Plasma glucose levels are shown as mean ± SEM for each group at different time points. All of the groups have reached the target hypoglycemic level (3±0.5 mmol/l blood glucose) from t=70min.
Figure 3-2. Glucose infusion rates during hyperinsulinemic hypoglycemic clamp experiments. The Non Diabetic SSTR2a, Non Diabetic Vehicle and the Diabetic SSTR2a required very low to no glucose to become and remain hypoglycemic at the target level of 3±0.5 mmol/l blood glucose. In contrast, the Diabetic Vehicle group had very high glucose requirements in order to maintain the hypoglycemia at the target level of 3±0.5 mmol/l blood glucose.
Figure 3-3. Insulin infusion rates distribution between the four groups during hyperinsulinemic hypoglycemic clamp experiments. The Diabetic Vehicle, Diabetic SSTR2a and the Non Diabetic Vehicle groups received 20 or 30 mU/Kg/min Insulin infusion via the jugular vein in order to induce and maintain the target hypoglycemic level during the clamp experiments. The mean ±SEM insulin infusion rates were not significantly different between the Diabetic Vehicle (25±1.89), Diabetic SSTR2a (25.56±1.75) and Non Diabetic Vehicle (26±2.44) groups; however we had to infuse more insulin at 50 mU/Kg/min for the animals in the Non Diabetic SSTR2a group in order to induce the target hypoglycemic. Data are shown as the individual insulin infusion rates for each animal in each group along with the mean ± SEM for each group.
Figure 3-4. A. Plasma glucagon levels during hyperinsulinemic hypoglycemic clamp experiments. Data are shown as mean ± SEM of glucagon secretion for each time point for different groups. B. Area under curve analysis of glucagon secretion in plasma during the hypoglycemic period from t=60 to t=120 min. Data are shown as mean ± SEM of AUCs for each group. Glucagon secretion during hypoglycemia is increased in Diabetic SSTR2a group by almost 2.3 folds compared to Diabetic Vehicle group (p=0.005), and this increase shows full restoration to the same level as the Non Diabetic Vehicle group (p=0.94). Otherwise, glucagon secretion during hypoglycemia is much blunted in Diabetic Vehicle group compared to the Non Diabetic Vehicle group (p<0.0001). SSTR2a treatment also enhanced the glucagon secretion in Non Diabetic SSTR2a group by nearly 3.3 folds compared to Non Diabetic Vehicle group, Diabetic SSTR2a and Diabetic Vehicle groups (p<0.0001).

(***: p<0.001 and **: p<0.01)
Figure 3-4. B.

AUC

SSTR2a

Diabetic Non-diabetic
Figure 3-5. A. Plasma corticosterone levels during hyperinsulinemic hypoglycemic clamp experiments. Data are shown as mean ± SEM of corticosterone secretion for each time point for different groups. B. Area under curve analysis of corticosterone secretion in plasma during the hypoglycemic period from t=60 to t=120 min. Data are shown as mean ± SEM of AUCs for each group. Corticosterone secretion in Diabetic Vehicle is not blunted and in fact increased during hypoglycemia, compared to Non Diabetic Vehicle group (p=0.009). SSTR2a treatment in Diabetic SSTR2a group resulted in enhancement of corticosterone secretion compared to Diabetic Vehicle (p=0.019), Non Diabetic Vehicle (p=0.0041) and Non Diabetic SSTR2a (p=0.006). Corticosterone secretion remained unchanged in Non Diabetic SSTR2a group compared to Non-Diabetic Vehicle group (p=0.412). (**: p<0.01 and *: p<0.05)
Figure 3- 5. B.

AUC
Figure 3-6. **A. Plasma epinephrine levels during hyperinsulinemic hypoglycemic clamp experiments.** Data are shown as mean ± SEM of epinephrine secretion for each time point for different groups. **B. Area under curve analysis of epinephrine secretion in plasma during the hypoglycemic period from t=60 to t=120 min.** Data are shown as mean ± SEM of AUCs for each group. Epinephrine secretion remained unchanged by the SSTR2a treatment in Diabetic (p=0.975) and Non Diabetic (p=0.249) groups. Also, the epinephrine response is not blunted in Diabetic vehicle compared to Non Diabetic Vehicle (p=0.412)
Figure 3-6. B.

![AUC graph showing the comparison between Diabetic and Non-diabetic groups for SSTR2a positive and negative conditions](image)

- **AUC**
- **SSTR2a**
- **Diabetic**
- **Non-diabetic**
Figure 3-7. A. Plasma norepinephrine secretory levels during hyperinsulinemic hypoglycemic clamp experiments. Data are shown as mean ± SEM of norepinephrine secretion for each time point for different groups. B. Area under curve analysis of norepinephrine secretion in plasma during the hypoglycemic period from t=60 to t=120 min. Data are shown as mean ± SEM of AUCs for each group. Norepinephrine secretion remained unchanged by the SSTR2a treatment in Diabetic (p=0.626) and Non Diabetic (p=0.059) groups. Also, the norepinephrine response is not blunted in Diabetic vehicle compared to Non Diabetic Vehicle (p=0.126)
Figure 3-7. B.
Figure 3-8. A. Plasma somatostatin levels during hyperinsulinemic hypoglycemic clamp experiments. Data are shown as mean ± SEM of somatostatin secreted in plasma for each time point for different groups. B. Area under curve analysis of somatostatin secretion in plasma during the hypoglycemic period from t=60 to t=120 min. Data are shown as mean ± SEM of AUCs for each group. Plasma somatostatin remained unchanged during hypoglycemia in Diabetic SSTR2a group compared to Diabetic Vehicle (p=0.735) and also in Diabetic Vehicle group compared to Non Diabetic Vehicle (p=0.173). SSTR2a treatment increased the plasma somatostatin during hypoglycemia in Non Diabetic SSTR2a group compared to Non Diabetic Vehicle, Diabetic Vehicle and Diabetic SSTR2a groups (p<0.0001). (**: p<0.001)
Figure 3- 8. B.
Figure 3-9. Whole pancreatic glucagon protein contents following hyperinsulinemic hypoglycemic clamp experiments. Data are shown as mean ± SEM of the whole pancreatic glucagon normalized to the total protein content of the pancreata. Diabetic SSTR2a group had a significantly lower pancreatic glucagon protein content compared to Diabetic Vehicle (p=0.0009), Non diabetic Vehicle (p=0.009) and Non Diabetic SSTR2a (p=0.002) groups. There was no significant difference between the pancreatic glucagon content of Diabetic Vehicle, Non Diabetic SSTR2a and Non Diabetic Vehicle groups. (***: p<0.001 and **: p<0.01)
Figure 3-10. Whole pancreatic somatostatin protein content following hyperinsulinemic hypoglycemic clamp experiments. Data are shown as mean ± SEM of the whole pancreatic somatostatin normalized to the total protein content of the pancreata. The pancreatic somatostatin content following hypoglycemia was slightly lower in Diabetic SSTR2a group compared to Diabetic Vehicle; however, the difference was not significant (p=0.057). SSTR2a treatment elevated the total pancreatic somatostatin content in the Non Diabetic SSTR2a group compared to Non Diabetic Vehicle; however, the difference was not significant (p=0.055). Diabetic Vehicle and Diabetic SSTR2a had lower pancreatic somatostatin contents compared to Non Diabetic SSTR2a group (p=0.002 and 0.0003 respectively). Also, while the pancreatic somatostatin content was not different between the Diabetic Vehicle and the Non Diabetic Vehicle (p=0.102), SSTR2a treatment significantly decreased the pancreatic somatostatin content following hypoglycemia in Diabetic SSTR2a group compared to Non Diabetic Vehicle (p=0.004). (**: p<0.01 and ***: p<0.001)
Figure 3-11. The whole pancreatic glucagon protein contents of BBDP rats in absence of hypoglycemia. Data are shown as mean ± SEM of the whole pancreatic glucagon normalized to the total protein content of the pancreata. The insulin-treated diabetic BBDP rats had a significantly lower pancreatic glucagon protein content compared to untreated diabetic and non diabetic BB rats (p=0.005 and 0.003 respectively). There was no significant difference between the pancreatic glucagon content of untreated diabetic rats and the non diabetic rats (p=0.827) (**: p<0.01)
Figure 3-12. The whole pancreatic somatostatin protein contents of BBDP rats in absence of hypoglycemia. Data are shown as mean ± SEM of the whole pancreatic somatostatin normalized to the total protein content of the pancreata. The insulin-treated diabetic BBDP rats had a significantly lower pancreatic glucagon protein content compared to untreated diabetic and non diabetic BB rats (p=0.0006 and 0.004 respectively). Although the pancreatic somatostatin content was slightly higher in untreated diabetic rats compared to the non diabetic rats, the difference was not statistically significant (p=0.277)

(***: p<0.001 and **: p<0.01)
Figure 3-13.A. Glucagon secretion from diabetic BBDP rat pancreatic slices. Data are shown as the mean ± SEM of glucagon secreted from the slices normalized to the total glucagon content of the slices for each group during the perifusion. B. Area under curve analysis of the glucagon secretion. Data are shown as mean ± SEM of AUCs for each group. SSTR2a treatment has significantly increased the glucagon secretion by approximately 2 folds at 1mmol/l glucose (p=0.015), 7mmol/l glucose (p=0.047) and 7mmol/l glucose+ 20 mmol/l arginine (p=0.003) conditions compared to control. (**: p<0.01 and *: p<0.05)
AUC of Glucagon secretion
Diabetic BB pancreatic slices perfusion

- + SSTR2a
- - SSTR2a

Glucacon secretion

1mM Glucose  7 mM Glucose  7mM Glucose + Arginin

* *
Figure 3-14.A. Glucagon secretion from Non diabetic BBDP rat pancreatic slices. Data are shown as the mean ± SEM of glucagon secreted from the slices normalized to the total glucagon content of the slices for each group during the perifusion. B. Area under curve analysis of the glucagon secretion. Data are shown as mean ± SEM of AUCs for each group. SSTR2a treatment increased the glucagon secretion by approximately 2.8 folds in 1mmol/l glucose (p=0.001), 7mmol/l glucose (p=0.009) and 7mmol/l glucose+ 20 mmol/l arginine (p=0.015) conditions compared to control. (**: p<0.01 and *: p<0.05)
AUC of Glucagon secretion
Non Diabetic BB pancreatic slices perifusion

- + SSTR2a
- - SSTR2a

Gulucagon secretion

1 mM Glucose  + -  7 mM Glucose  + -  7 mM Glucose + Arginin  + -

*** ** *
Figure 3-15. Glucagon and insulin secretion from perifused human pancreatic slices. A. Data shows the mean ± SEM of glucagon secreted from the pancreatic slices in different conditions normalized to the total glucagon content of the slices. B. Data shows the mean ± SEM of insulin secreted from the pancreatic slices in the different conditions normalized to the total insulin content of the slices. Since the N for experiments was 2, statistical analysis was not performed.
Figure 3-15. B.

Human Pancreatic Slices Insulin Secretion in Perifusion (N=2)
Chapter 4 Discussion

It is well established that the glucagon counterregulatory response to hypoglycemia is compromised in type 1 diabetes (Cryer, 2008a, 2008b). There is a wealth of evidence regarding the inhibitory effect of somatostatin on glucagon secretion from pancreatic α-cells; there are also numerous studies suggesting a local paracrine inhibitory effect by pancreatic somatostatin (Brunicardi, et al., 1994; Cejvan, et al., 2003; Itoh, et al., 1980; Kleinman, et al., 1994; Salehi, et al., 2007; M. Z. Strowski, et al., 2000). It is also well established that somatostatin induces its inhibitory effects on α-cells via SSTR2 in humans (Singh, et al., 2007; Mathias Z. Strowski & Blake, 2008) and rodents (Cejvan, et al., 2003; M. Z. Strowski, et al., 2000).

It had been proposed that the excessive somatostatin levels observed in type 1 diabetic human (Segers, et al., 1989), rodents and dog plays a role in the impairment of glucagon counterregulation response to hypoglycemia (Rastogi, et al., 1990; J. T. Yue, et al., 2012). Considering the exclusive role of SSTR2 on pancreatic α-cells in mediating the inhibitory effects of somatostatin on glucagon secretion, it was hypothesised that antagonising SSTR2 would improve glucagon counterregulation in response to hypoglycemia (J. T. Yue, et al., 2012). This hypothesis was first tested in STZ diabetic rats and it was observed that antagonising SSTR2 fully restores the glucagon and corticosterone counterregulation to insulin-induced hypoglycemia in STZ diabetic rats (J. T. Yue, et al., 2012). The STZ model is however not a genuine disease model of humans
in which T1D is an autoimmune disorder. Thus in the current study, I had employed a well accepted autoimmune T1D model that resembled human T1D, and that is the diabetic BBDP rat (Bortell & Yang, 2012). In the present study for the first time we demonstrate that antagonising the SSTR2 in insulin treated autoimmune type 1 diabetic BBDP rats, fully restores the glucagon counterregulation to insulin induced hypoglycemia and indeed, increasing the glucagon secretion during hypoglycemia by 2.3 folds.

The autoimmune type 1 diabetes which occurs in the BBDP rats closely parallels human type 1 diabetes in the most aspects compared to the other animal models of type 1 diabetes and it is often used to investigate the potential intervention therapies for clinical trials (Bortell & Yang, 2012). A most important feature of this model mimicking human T1D, in addition to the autoimmune origin of diabetes, is the requirement of continuous exogenous insulin therapy in these animals in order to prevent the lethal complications of type 1 diabetes. Thus, we can assume that the hyperinsulinemic hypoglycemic clamp in this model closely mimicked the iatrogenic hypoglycemia in type 1 diabetes, simulating the common situation in the diabetic patients who receive medications that raise the circulating plasma insulin (Cryer, 2012a) as discussed previously in the introduction.

We also observed that SSTR2a treatment enhances the glucagon secretion during insulin induced hypoglycemia in non diabetic BBDP rats, however the previous study on normal Sprague Dawley did not showed any enhancement of glucagon secretion by SSTR2a treatment, this could be probably because the non diabetic BBDP is not really normal, but at a subclinical stage that could proceed to diabetes. Histologically, the non
diabetic BBDP pancreatic islets were observed also to be inflamed, and possibly some inflammatory mediators might have undefined effects on alpha cells to have sensitized to somatostatin inhibition.

To be consistent with the previous studies in the field, we also measured the epinephrine, norepinephrine and corticosterone responses to hypoglycemia after SSTR2a treatment. We did not observe any change in epinephrine and norepinephrine responses by SSTR2a treatment; however, we did observe enhanced corticosterone response to hypoglycemia. This finding is of particular importance; as in the clinical settings during the prolonged hypoglycemia especially during sleep in children with type 1 diabetes, cortisol response is significant and the current results suggest that SSTR2a treatment may enhance the cortisol response during hypoglycemia in type 1 diabetes. The epinephrine, norepinephrine and corticosterone responses were not blunted in the Diabetic Vehicle group, suggesting that these responses are not affected in insulin treated type 1 diabetic BBDP rats at least early in the time course of the disease, as has been previously proposed (Jacob, Dziura, Morgen, Shulman, & Sherwin, 1996). The other possibility may be that as we have studied the insulin treated diabetic rats in just 2 weeks from the onset of diabetes; the time course of diabetes in these animals were likely too short to compromise these counterregulatory responses to hypoglycemia observed in latter stages of the disease. As previously discussed in the Introduction, the prior glycemic control and the time course of the diabetes with regard to recurrent hypoglycemic episodes in type 1 diabetic models are important factors affecting
nor/epinephrine and corticosterone counterregulation (Chan, et al., 2006; M. Davis, et al., 1994; Kinsley & Simonson, 1996), which were not tested in my study.

We demonstrated that the pancreatic glucagon following hypoglycemia is not decreased in the Diabetic Vehicle rats when compared to the Non-Diabetic Vehicle rats, thus the defect in glucagon counterregulation may not be due to a decrement in glucagon synthesis in autoimmune type 1 diabetes; this finding is well in line with the previous study on the STZ diabetic rats that also showed unaffected pancreatic glucagon content in diabetes following hypoglycemia (J. T. Yue, et al., 2012). SSTR2a administration decreased the pancreatic glucagon protein content, while the secretory glucagon levels in the plasma in response to hypoglycemia were fully restored. This finding suggests that antagonizing the SSTR2 may increase α-cells potency to release glucagon in response to hypoglycemia.

We observed that the pancreatic somatostatin protein content in the untreated diabetic rats is increased; however, it did not reached to a statistical significant higher level compared to the non-diabetic rats. Furthermore, our results indicate that insulin treatment significantly decreases the pancreatic somatostatin level in the diabetic BBDP rats, which is consistent with the previous studies suggesting that insulin treatment corrects the elevated pancreatic somatostatin levels in type1 diabetes (Papachristou, et al., 1989; Patel, et al., 1983; Rastogi, et al., 1990). The fact that the insulin treated diabetic BB rats don’t have elevated pancreatic somatostatin and yet SSTR2a can normalize hypoglycemia, demonstrates that the problem occurs not only when somatostatin is increased, but also when it is normal. We would assume that in humans, where
somatostatin is increased, the effect would be even more marked. We also observed that while the pancreatic somatostatin protein content remained unchanged following the insulin induced hypoglycemia in Diabetic SSTR2a group compared to the Diabetic Vehicle group, the plasma levels of somatostatin were slightly higher in the Diabetic SSTR2a compared to the Diabetic Vehicle group. This finding suggests that the increased plasma somatostatin during hypoglycemia may be originated from the extra pancreatic sources particularly the gastrointestinal system (Patel, et al., 1983; Patel, Wheatley, Malaise-Lagae, & Orci, 1980; Ruggere & Patel, 1984). The same results for the Non Diabetic SSTR2a group, further confirms the extra pancreatic origin of the increased plasma somatostatin during hypoglycemia in this group. Nevertheless, the increased pancreatic somatostatin content in Non Diabetic SSTR2a group, immediately after hypoglycemia, may indicate the compensatory increase in somatostatin synthesis resulted from blocking its action by antagonising the SSTR2.

The compensatory increase in somatostatin synthesis and secretion in Non-diabetic SSTR2a group may be one reason behind the requirement of more exogenous insulin needed in this group in order to attain hypoglycemic during the clamp experiments. The increased levels of somatostatin in Non-Diabetic SSTR2a group, further inhibits the endogenous insulin secretion and increases the exogenous insulin requirements to induce the target hypoglycemia (3±0.5 mmol/l). Indeed, we postulate that the main reason for the high exogenous insulin requirement for Non-Diabetic SSTR2a group is the enhanced plasma glucagon levels in this group as the result of SSTR2 blockade.
It has been postulated that the pancreatic somatostatin regulates glucagon secretion by a local paracrine action (Unger & Orci, 1977). This notion has been supported by the studies demonstrating the enhanced glucagon and insulin secretion by neutralizing the intra-islet somatostatin by monoclonal antibodies in perfused human pancreas (Brunicardi et al., 2001; Kleinman et al., 1995). Developing the specific SSTR2 antagonist (Hocart, et al., 1999) opened the way to investigate the paracrine somatostatin action on glucagon and insulin action more accurately. It was shown that administration of PRL-2903, the same SSTR2a that has been used in the current study, enhances the arginine-stimulated glucagon secretion from the perifused rat isolated pancreatic islets and perfused isolated pancreata (Cejvan, et al., 2003). Also it has been shown that SSTR2a reverses the effects of SSTR2 specific agonist on insulin and glucagon secretion in a dose response manner in perifused isolated human pancreatic islets (Singh, et al., 2007). Developing somatostatin and SSTR2 knockout mice (Hauge-Evans, et al., 2009; M. Z. Strowski, et al., 2000) also further confirmed the paracrine tonic inhibitory effect of pancreatic somatostatin on insulin and glucagon secretion from β and α-cells.

In the present study to further investigate the local effect of the pancreatic somatostatin on glucagon secretion and also to further explore the SSTR2a effect on enhancing and restoring the perturbed glucagon counterregulatory response to hypoglycemia in type 1 diabetes, we developed a new method. We deployed the previously introduced fresh thin pancreatic slices (Huang, et al., 2011; Speier & Rupnik, 2003) in the perifusion setting. Employing this strategy, we could address the real-time glucagon secretory responses to hypoglycemia in both healthy and diabetic conditions.
devoid of the other intervening factors present in-vivo like the effects of the central and autonomic nervous systems. This would not have been possible by employing the conventional methods, as the pancreatic islets after T1D autoimmune injury and scarring are considerably smaller in size, and much distorted shape and architecture, and any attempt to isolate them by exposing them to enzymatic digestion will further damage them (Huang, et al., 2012). Furthermore, since α-cells are located mostly in the islet periphery in rodents (Cabrera, et al., 2006), they would be even more prone to injury from the isolation procedures. In case of whole pancreatic perfusion, the interference of the nervous system cannot be ruled out.

In the pancreatic slices perifusion studies, we observed that antagonizing the SSTR2 significantly enhances the glucagon response during hypoglycemia both in diabetic and non-diabetic BDBP rats. This finding further confirms the paracrine inhibitory effect of pancreatic somatostatin on glucagon secretion which is mediated via the SSTR2 on α-cells. In addition, we also observed that the basal glucagon secretion is enhanced in both diabetic and non-diabetic animals.

There is abundance of studies suggesting that the clinical manifestations of type 1 diabetes are the result of the hyperglucagonemia which contributes to the hyperglycemia (Lee et al., 2012; Lee, Wang, Du, Charron, & Unger, 2011; Unger & Cherrington, 2012). Considering SSTR2a as a potential potent therapeutic for preventing hypoglycemia in type 1 diabetes, by augmenting the glucagon secretion through antagonising the SSTR2 on α-cells, it is very important to investigate its effect on basal glucagon secretion. The previous study on STZ diabetic rats well indicated that SSTR2a infused for 4 hours
during basal conditions in-vivo only resulted in a transient glucagon increase which neither induced hyperglycemia nor altered corticosterone and catecholamine levels in the plasma (J. T. Yue, et al., 2012).

The observed initial increase in basal glucagon secretion in the perifused pancreatic slices by SSTR2a treatment may be because of the inherent limitation of our study. Nonetheless, our in-vitro studies indicate that blockade of the actions of endogenous pancreatic somatostatin on alpha cells particularly on its SSTR2, devoid of the other confounding factors possibly present in-vivo which could prevent the hyperglucagonemia induced hyperglycemia under basal conditions.

Previously it was shown that SST2a reversed the inhibitory effects of specific SSTR2 agonist on insulin and glucagon secretion in a dose response manner in isolated human islets (Singh, et al., 2007); however the effects of SSTR2a on enhancing the glucagon secretion in hypoglycemia was never tested in human pancreas. Employing the thin pancreatic slices perifusion technique, for the first time we demonstrate that SSTR2a enhances the glucagon secretory response to hypoglycemia stimulus in healthy human pancreatic slices. Our results further demonstrate that the simultaneous insulin secretion from the human pancreatic slices during hypoglycemia was also enhanced by the SSTR2 blockade. This could be explained by the observations of other groups who have suggested that SSTR2 is the functionally predominant somatostatin receptor on both α and β-cells in human pancreas which mediates the somatostatin actions on these cells (Brunicardi, et al., 2003; Kailey, et al., 2012; Moldovan, et al., 1995).

We are confident that our technical approach yielded an accurate estimate of glucagon secretion from the pancreatic slices in the perifusion protocol which we
observed to be enhanced when the slices were treated either with SSTR2a or arginine; and as well, also showed a downward trend in transition from 1 mmol/l to 7mmol/l glucose concentration in the Non-Diabetic and Diabetic BBDP pancreatic slices. Thus, our results are well in line with the previous reports, suggesting that glucose has the highest inhibitory effect on glucagon release from α-cells at 6-7 mmol/l glucose concentrations (Salehi, et al., 2006; Vieira, et al., 2007; Walker et al., 2011).

Overall, the response differences between pancreatic slices perifusion in-vitro and the in-vivo experiments are due to the fact that the slices are not innervated, but also because there is no blood flowing through the slices. For example, blood contains arginine, and as indicated through the results, arginine by itself stimulates glucagon release. Thus, we further hypothesize that since hypoglycemia by itself does not stimulate glucagon release, it is possible that glucagon release will be stimulated when hypoglycemia is accompanied by arginine and other amino acids. It’s also possible that during hypoglycemia, blood flow through the islets changes and also hypoglycemia can stimulate release of many amino acids.

The potent effects of the SSTR2a on normal human pancreas maybe because the population of D-cells in human islets are probably twice that of rodent islets, whereas β-cells populations at 55% are less than rodents at 75% (Cabrera, et al., 2006). This would suggest that the proportionate inhibitory effects of insulin and somatostatin on human α-cells may be different than rodents, with somatostatin probably having greater effect on human alpha cells. Hence, SSTR2a actions were more evident on the normal human alpha cells and not on normal Sprague Dawley rats. This has a strong clinical implication in that human type 1diabetic patient with destroyed beta cells, leaving a potentially larger
D-cell population would predictably respond better to SSTR2a blockade than rodent type 1 diabetic models.
Chapter 5 Conclusion and future directions

We had hypothesised that the impaired glucagon counter-regulation in type 1 diabetes is due to the accentuated somatostatin inhibitory effect on α-cells in the absence of endogenous insulin; thus antagonising the somatostatin receptor type 2, in order to block the somatostatin inhibitory effect on α-cells would improve the compromised glucagon counter-regulatory response to hypoglycemia in autoimmune type 1 diabetic rats. In the present study we demonstrate for the first time that antagonising the SSTR2 in insulin treated autoimmune type 1 diabetic BBDP rats, fully restores the glucagon counterregulation to insulin induced hypoglycemia and indeed, increasing the glucagon secretion during hypoglycemia by 2.3 folds. This proves our hypothesis that somatostatin suppression of alpha cells in type 1 diabetes contributes to the impaired glucagon secretory response to hypoglycemia.

The present findings further suggest that SSTR2 antagonism can enhance hypoglycemia-stimulated glucagon and corticosterone release sufficient to restore normoglycemic control in type 1 diabetic BB rats.

In the present study we further investigated the local effect of the pancreatic somatostatin on glucagon secretion and also explored the SSTR2a effect on enhancing and restoring the perturbed glucagon counterregulatory response to hypoglycemia in type 1 diabetes in BB diabetic rats by employing the pancreatic slices perifusion technique.
This is the first time that the thin pancreatic slices (Huang, et al., 2011; Huang, et al., 2012; Speier & Rupnik, 2003) are employed perifusion studies. This study would not have been possible employing the conventional methods, as the pancreatic islets after T1D autoimmune injury and scarring are considerably smaller in size, and much distorted shape and architecture, and any attempt to isolate them by exposing them to enzymatic digestion will further damage them (Huang, et al., 2012).

In the pancreatic slices perifusion studies, for the first time we demonstrate that antagonizing the SSTR2 significantly enhances the glucagon response during hypoglycemia both in diabetic and non-diabetic BBPD rats. This finding further confirms the paracrine inhibitory effect of pancreatic somatostatin on glucagon secretion which is mediated via the SSTR2 on α-cells.

Benefiting from this novel technical approach, the present work is the first study to show the improvement in glucagon secretion from the normal human pancreatic slices by antagonizing the somatostatin receptor type 2 on α-cells by a specific SSTR2a (PRL-2903).

These results suggest that SSTR2 blockade may be a novel treatment strategy to improve the counter-regulatory responses, particularly of glucagon in type 1 diabetes. SSTR2 antagonism should be explored to be ascertained that in patients with type 1 diabetes, the impaired glucagon and cortisol secretion during hypoglycemia can be restored; this could be an effective treatment to prevent the acute and chronic complications of hypoglycemia. Also by minimizing the risk of hypoglycemia, type 1
diabetic patients could benefit from intensive insulin treatment which ultimately would lead to a lower incidence of diabetic complications.

In the future, we are also aiming to explore factors in the circulation that are necessary to sensitize the response of α-cell to hypoglycemia and the mechanism of the potential sensitization of α-cells to insulin in diabetes (Gaisano, et al., 2012).

Since the slice preparation has enabled us to begin to assess α-cell dysfunction in type 1 diabetes where in the very small islet mass and inflammation would have rendered it impossible to reliably isolate and examine the α-cell (Gaisano, et al., 2012; Huang, et al., 2012), in future we are planning to examine the precise action of SSTR2a on α-cell, employing the pancreatic slices in electrophysiologic studies to see which ion channel or exocytotic pathways might be affected (Huang, et al., 2011; Huang, et al., 2012). In our recent study (Huang, et al., 2012) in fact, we found that α-cell of STZ-treated mice, rendered T1D, there was perturbation of two ion channels, an increase in Na channel density and reduction of voltage-gated K+ channel density, which contributed to the increased action potential firing frequency and amplitude, coupled with larger glucagon granules (by electron microscopy), together rendering the α-cell primed to secrete more glucagon; hence revealing the elusive basis of hyperglucagonemia. It would be of interest to see if similar ion channels in BB rat α-cells are perturbed, and if SSTR2 blockade then acts on the primed α-cell to stimulate more glucagon secretion. The major distinction between the STZ models and the BB rat model is that the latter is an autoimmune model, and hence involved a myriad of inflammatory processes. It would
be very exciting to test if any one of the inflammatory mediators might play a role in modulating $\alpha$-cell sensitivity for glucagon release.

Another strategy to explore this nature of glucagon secretion restored by the SSTR2 blockade is to directly examine glucagon exocytosis employing multi-photon imaging, used extensively to examine beta-cell exocytosis (Takahashi, Ohno, & Kasai, 2012), in order to precisely investigate the real time glucagon secretion from $\alpha$-cells induced by SSTR2a blockade. This is actually unprecedented and the precise exocytotic events in $\alpha$-cells are unknown. The major advantage of this approach is that we would be able to subject the pancreatic slice to physiologic stimulus i.e. different glucose concentrations as I have done to examine glucagon secretion on the slices; and then assess the precise exocytotic event(s) that is modulated by the SSTR2 blockade. The electrophysiologic study, which will reveal the precise ion channel events specifically altered by somatostatin in disease, is nonetheless not a physiologic assay. Thus the combination of both the exocytotic imaging and electrophysiologic assays will determine the underlying mechanism of the effects of somatostatin and SSTR2a blockade on modulating glucagon secretion in T1D that I have observed in this in vivo study.

Since we have demonstrated the enhancement of glucagon secretion by antagonizing SSTR2 in the perifused thin human pancreatic slices, it would be of particular interest to perform some of the above exocytotic imaging and electrophysiologic studies on $\alpha$-cells within human pancreas slices.

Finally, we need to put all these observations into the clinical perspective, which is how these insights would better the lives of patients with type1diabetes. It would
therefore be of great interest to proceed to a small clinical study to see if SSTR2 blockade might work on T1D patients in preventing exogenous insulin induced hypoglycemia by restoring low-glucose stimulated glucagon secretory response. In fact, this ‘discovery’ has been patented by Professor Mladen Vranic, my co-supervisor to precisely and eventually carry out such studies on diabetic patients.
Chapter 7 References


