# 4. DISCUSSION

Insulin and glucagon regulate glucose homeostasis. After food intake, the secretion of insulin is stimulated while glucagon secretion is inhibited. Increased postprandial blood glucose is cleared through uptake by the liver, muscle and adipocytes.

Physiologically, the secretion of glucagon is promoted during fasting state. Glucagon raises blood glucose concentration due to stimulation of hepatic glycogenolysis and gluconeogenesis (Cherrington et al., 1981) (Magnusson et al., 1995).

The secretion of SST is stimulated following food ingestion. SST inhibits both insulin and glucagon secretion. The action of somatostatin on the target cells is mediated via five receptor subtypes (SSTR1- SSTR5), which belong to G protein-coupled receptor family.

The function of SSTRs in the regulation of insulin and glucagon secretion in rodents is well known (Rossowski and Coy; 1994) (Fagan et al., 1998) (Strowski et al., 2000) (Cejvan et al., 2003) (Strowski et al., 2003). On the contrary, the role of SSTRs in regulating insulin and glucagon secretion in humans is not well characterized. Studies investigating the expression pattern of SSTRs in the endocrine pancreas provided discrepant results (Atiya et al., 1997) (Moldovan et al., 1995) (Zambre et al., 1999). The discrepancies could be due to the use of moderate selective SSTRs agonists in earlier studies or due to different experimental setups.

In this study, freshly isolated, high quality of pancreatic islets derived from healthy cadavers were used. In addition, highly SSTR-selective agonists (Rohrer et al., 1998) (Rohrer et al., 2000) were used.

This study suggest that the expression pattern of SSTRs reported do not correspond with their functions in humans (Kumar et al., 1999). SSTR2 was the most potent inhibitor of both insulin and glucagon secretion, although only 46 % of β-cells express SSTR2. Nearly 100 % of β-cells expressed SSTR5, followed by SSTR1, which was expressed by 87 % of these cells (Kumar et al., 1999). The present study suggests, beside SSTR2, SSTR5 was more potent inhibitor of insulin secretion compared to SSTR1, despite SSTR1 having a higher expression level as compared to SSTR5. An explanation for this discrepancy remains speculative.

Despite the less abundant expression of SSTR2 compared to SSTR5, SSTR2-selective agonist more potently inhibited insulin secretion as compared to the SSTR5-selective agonist.

The *in vitro* data of this study suggest that SST regulates insulin secretion from human pancreatic islets mainly via SSTR2 and SSTR5, and less potently via SSTR1. The involvement of SSTR2 at inhibiting insulin secretion from isolated human pancreatic islets was confirmed by using a highly SSTR2-selective antagonist DC-41-33. The inhibitory effect of the SSTR2-selective agonist on insulin secretion was reversed by DC-41-33 in a dose-dependent manner.

The data confirms the major role of SSTR2 in regulation of insulin secretion in humans. The present study shows that the regulation of insulin secretion by SSTRs do not follow the SSTR expression pattern (Kumar et al., 1999). Similar to the present study, couple of previous studies have described, SSTR2 as an inhibitor of insulin secretion on perfused human pancreas (Atiya et al., 1997) (Brunicardi et al., 2003). Since these studies were performed on perfused human pancreas, inhibition of insulin secretion could be due to other factors which also express SSTR2. However, in these studies less selective SSSTR2 agonists were used.

Inconsistent with our results, Zambre et al., (Zambre et al., 1999) have observed that SSTR2 was unable to inhibit insulin secretion in isolated human pancreatic islets, although SSTR5 was effective. The potential explanation could be the use of a less selective SSTR2 agonist.

Sofar it is not known which SSTR inhibits glucagon secretion in humans. Earlier studies demonstrated that nearly 89 % of human α-cells express SSTR2, followed by SSTR5 (35 %) and SSTR1 (26 %) (Kumar et al., 1999). Similar to insulin secretion, we observed that SSTR2 was the most potent inhibitor of glucagon secretion from isolated human pancreatic islets, whereas SSTR1 and SSTR5 were less effective.

The role of SSTR2 in inhibiting insulin and glucagon secretion was again confirmed by using highly selective SSTR2 antagonist DC-41-33. This work demonstrated for the first time the role of SSTR2 as a potent inhibitor of both insulin and glucagon secretion from isolated human pancreatic islets. The SSTR5-selective agonist also showed an inhibition of insulin and glucagon secretion although SSTR5 was less abundantly expressed than

SSTR1. SSTR1 also plays a role in inhibitng glucagon secretion, despite its higher expression on pancreatic β-cells.

In summary, in isolated human pancreatic islets, the data suggest that insulin and glucagon secretion are inhibited mainly via SSTR2. SSTR5-selective agonist was more potent in inhibiting insulin secretion than glucagon secretion. SSTR1 showed higher potency in inhibiting glucagon secretion than insulin secretion in isolated human pancreatic islets.

In T2DM, the postprandial suppression of glucagon secretion is markedly impaired. Increased glucagon leads to hyperglycemia. Earlier study (Brand et al., 1994) demonstrated that immunoneutralization of endogenous glucagon by anti-glucagon antibodies alleviates hyperglycaemia. Using isolated human pancreatic islets, we observed that SSTR2 is involved in the inhibition of glucagon secretion. We therefore tested the role SSTR2 in regulating glucose homeostasis in mice with T2DM (Strowski et al., 2006) and in mice with diet-induced obesity (Singh et al., 2007). In the former study, highly selective SSTR2 non-peptidal agonist (compound 1) has been used which only inhibited glucagon secretion without affecting the insulin secretion. This effect was observed in a system of isolated murine pancreatic islets. The specificity and selectivity of the SSTR2-selective agonists was confirmed by the lack of suppression of circulating glucagon and blood glucose concentrations by this agonist in $SSTR2^{-/-}$ mice.

This study shows, for the first time that SSTR2 has a pivotal role in inhibition of glucagon secretion. This is important in the context that hyperglucagonemia increases blood glucose by promoting hepatic glucose production and output in T2DM. The non-peptidal highly SSTR2-selective agonist improves glucose control in animals with high fat diet-induced obesity and insulin resistance. Similar to individuals with T2DM, this study suggest an impaired inhibition of glucagon secretion by both exogenous glucose and SST in $SSTR2^{-/-}$ mice fed high fat diet (Shah et al., 1999) (Ohneda et al., 1978).

As reported in T2DM, hyperglycemia which is partly due to abnormally increased postprandial concentration of glucagon, was also observed in $SSTR2^{-/-}$ animals fed high fat diet. Impaired inhibition in glucagon secretion was registered in these mice challenged with high glucose load and exogenous SST. Continuous feeding with HFD results in peripheral insulin resistance and β-cell dysfunction. Since we observed that non-fasting

glucagon secretion was impaired in SSTR2$^{-/-}$ mice with DIO, we conclude that SSTR2 has a crucial role in the inhibition of glucagon secretion. These data suggest that inhibition of glucagon secretion is mediated by SSTR2.

In addition, SSTR2$^{-/-}$ not only showed the impaired glucose tolerance but also reduced deposition of glycogen in the liver. Interestingly, enzymes which are responsible for glycogen storage were down regulated while enzymes responsible for glycogen breakdown were up regulated in livers of SSTR2$^{-/-}$ mice. For example glucokinase (GK) an enzyme which facilitates phosphorylation of glucose to glucose-6-phosphate which is the first step for glycogen synthesis and glycolysis was down regulated in SSTR2$^{-/-}$ mice. Decrease in GK expression in liver of SSTR2$^{-/-}$ mice suggests that hepatic glucose uptake was reduced in these mice.

Hepatic glycogen metabolism is a tightly regulated process by two enzymes glycogen synthase (GS) and glycogen phosphorylase (GP) (Villar-Palasi et al., 1997) (Ferrer et al., 2003) (Postic et al., 2004). Both GS and GP are regulated by allosteric effectors and by phosphorylation mechanisms. GS is active in dephosphorylated form while GP is active in phosphorylated form **(Fig. 23)**. GS converts the excess of glucose into glycogen for the storage in liver, while GP breaks stored glycogen back into glucose subunits. Glucagon inhibits glycogen synthesis, through reduction of the activity of GS whereas it stimulates the activity of GP via cAMP/protein kinase-A (Villar-Palasi et al., 1997) (Ferrer et al., 2003) (Postic et al., 2004) GS is inactivated (phosphorylated) by the enzyme GSK-3, which is active in non-phosphorylated form. Likewise, data shows that the phosphorylated (non-active) levels of GSK-3 in SSTR2$^{-/-}$ mice were found lower, while that GS were higher compared to WT mice **(Fig. 23)**.

Similar to this, the levels of phosphorylase kinase (PhK) which is responsible for the activation of GP were higher in livers of SSTR2$^{-/-}$ mice compared to WT mice. This suggests that raised glucagon levels in SSTR2$^{-/-}$ mice correlate with the breakdown of hepatic glycogen **(Fig. 23)**.

Expression data suggest that glucagon storage as glycogen in liver in SSTR2-/- deficient mice is due to the decreased expression of enzymes responsible for glycogen synthesis and increased expression of enzymes responsible for glycogen breakdown.

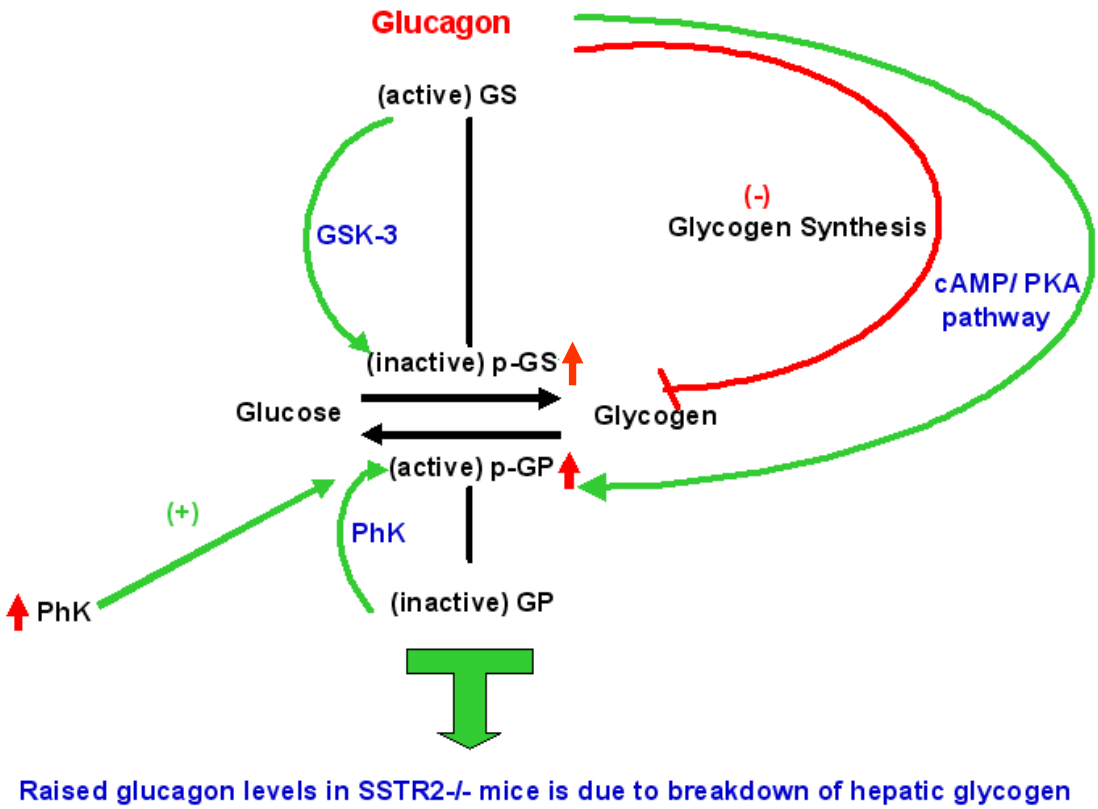


**Fig. 23.** Schematic representation of enzymes and pathways involved in hepatic glycogen control in SSTR2$^{-/-}$ mice.

Consistent with these results, the study suggest that the enzymes which are responsible for the production of glucose by gluconeogenesis (*de novo* glucose production) were up regulated in SSTR2$^{-/-}$ mice. PEPCK (phosphoenolpyruvate-carboxykinase) which is the rate limiting enzyme for gluconeogenesis is activated by glucagon through a CREB-dependent phosphorylation. This process is mediated via cAMP/PKA pathway (Gonzalez, Montminy; 1989) (Hanson, Reshef; 1997) (Dalle et al., 2004).

Conversion of pyruvate to glucose is the central pathway of gluconeogenesis. PEPCK converts oxaloacetate to phosphoenolpyruvate (PEP) which is the rate limiting steps of gluconeogenesis (Rognstad; 1979). **(Fig. 24)**.

In addition, G6Pase, which catalyses the final step of gluconeogenesis **(Fig. 24)** (production of glucose from glucose-6-phoshate) is stimulated by glucagon. As described above that glucagon activates enzymes which stimulate gluconeogenesis, in agreement to this, data of the present study shows similar effects. Data of the present study shows the increased levels of gluconeogenic enzymes CREB and PEPCK in SSTR2$^{-/-}$ deficient mice, while expression of G6Pase was not significantly increased.

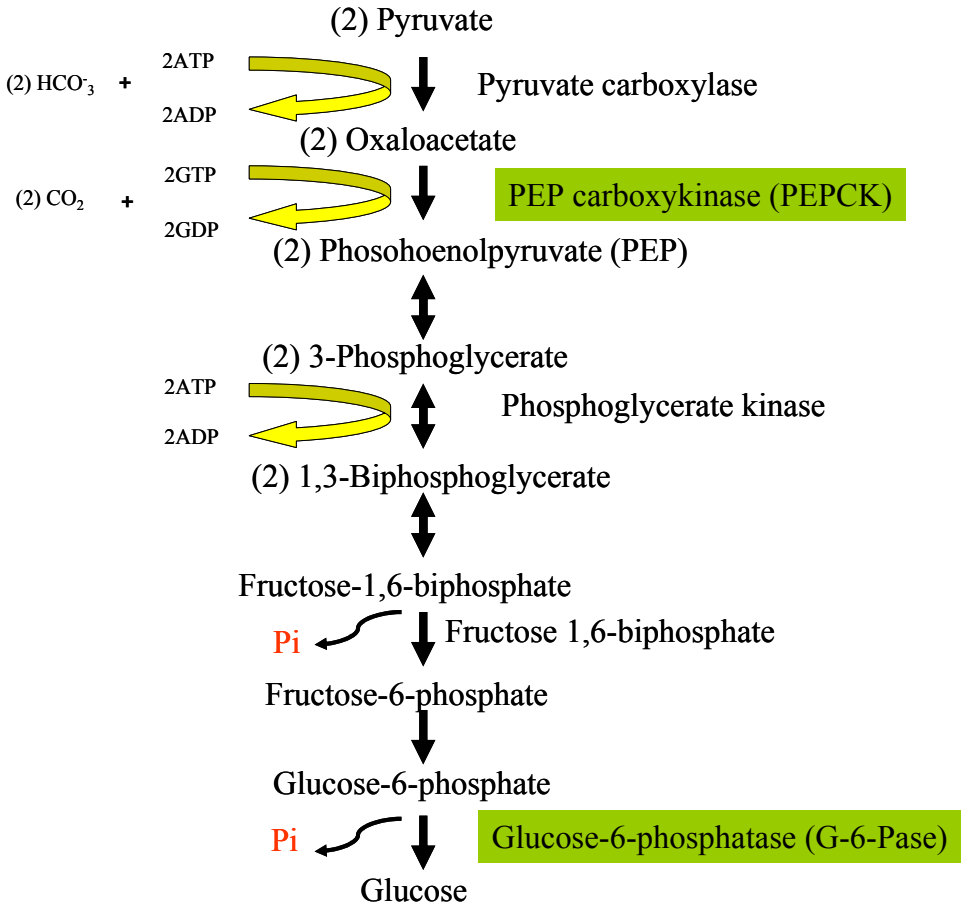


**Fig. 24.** Flow chart showing steps and enzymes involved in gluconeogenesis pathway
Adapted and modified from http://web.indstate.edu/thcme/mwking/gluconeogenesis.html

Deposition of fat and glycogen leads to fatty liver in patients with obesity or T2DM. (Van Steenbergen and Lanckmans; 1995). Hippen et al., has demonstrated that administration of glucagon alleviates fatty liver diseases (Hippen et al., 1999) and study on Gcg-ASO treated db/db mice strengthens the lipolytic action of glucagon. (Liang et al., 2004). Glucagon activates gluconeogenesis and fatty acid oxidation by stimulating cAMP-responsive transcription factor CREB (Herzig et al., 2003). The present study shows that $SSTR2^{-/-}$ mice which had high levels of circulating glucagon not only showed high expression of CREB but also a decrease of SREBP levels. SREBP is known to increase hepatic lipid accumulation (Shimano et al., 1996). The present study exploits an increase

in the expression of lipoprotein lipase, which could be responsible for the decrease in hepatic lipid content.

The present study shows that increase in lipolysis results in increase in the levels of non-esterified free-fatty acids (NEFA) in $SSTR2^{-/-}$ deficient mice. Increased levels of NEFA can impair the insulin sensitivity (Bevilacqua et al., 1987) and this was also observed in $SSTR2^{-/-}$ mice with DIO. The results suggest that increase levels of NEFA could be responsible for the increased blood glucose levels in $SSTR2^{-/-}$ mice fed high fat diet.

As previously mentioned administration of SSTR2 selective agonist (compound1) causes a decrease in glucose and glucagon in ob/ob mice without effecting the insulin secretion. This result suggests that activation of SSTR2 by selective agonists could lower glucose concentration in patients with T2DM.

To characterize the intracellular signalling of SSTR-dependent regulation of insulin and glucagon secretion, the following study to identify the *in vitro* effects of SSTR-selective agonists on insulin- and glucagon-producing cells was undertaken.

Interestingly, the study shows that SSTR2 is the only SSTR subtype expressed in glucagon producing InR1-G9 cells, which plays a potent role in the inhibition of glucagon secretion. SSTR2-selective agonist inhibited glucagon secretion in a time- and dose-dependent manner. Since SSTR2 reduces adenylate cyclase and inhibits calcium influx, these data suggest that these mechanisms may contribute to the inhibition of glucagon secretion. In summary, reduction of glucagon secretion by SSTR2-selective agonist may provide a novel strategy to lower hyperglucagonemia-induced hyperglycaemia in T2DM.

However, SSTR2 is not only involved in inhibition of glucagon secretion. SSTR2-selective agonist also inhibited insulin secretion from INS-1 cells. Data of the present study suggest that SSTR2-selective agonist was more potent in inhibition of high glucose- and exendin-4-stimulated insulin secretion than SSTR3 selective agonist.

Data shows that SSTR2 inhibited intracellular cAMP production. cAMP/protein kinase A-dependent pathway has been demonstrated to play a role in regulating insulin secretion in INS-1 cells (Ding et al., 2003). Agents (e. g. GLP-1) that increase cAMP production can increase insulin secretion and proinsulin gene expression (Drucker et al., 1987) (Fehmann & Habener; 1992) (Skoglund et al., 2000) (Kemp & Habener; 2002).

Both SSTR2 and SSTR3 selective agonists reduced the intracellular cAMP levels production in a dose-dependent way.

The secretion of both insulin and glucagon is regulated by $Ca^{2+}$. Influx of $Ca^{2+}$ activates insulin release. The present study shows that the blockade of R-type $Ca^{2+}$ channels by using highly selective R-type $Ca^{2+}$ channel blocker SNX-482 prevents the inhibition of insulin secretion from INS-1-cells by SST-14, and by agonist selective for SSTR2 and SSTR3. These data suggest that R-type $Ca^{2+}$ channel plays an important role in mediating the SST-inhibition of insulin secretion from the clonal beta cell line INS-1.

In addition, the present study shows the novel finding that SSTR2 inhibits insulin secretion via PI3-kinase pathway in INS-1 cells.

The study addresses no effect of SST-14 and SSTR2 selective agonist on total PDK1 and Akt protein levels, but a decrease in PDK1 and Akt phosphorylation levels which are the two downstream molecules of PI3-kinase pathway. PDK1 and Akt are active in phosphorylated form. Akt transduces its signals by phosphorylating and activating or inactivating a number of substrates.

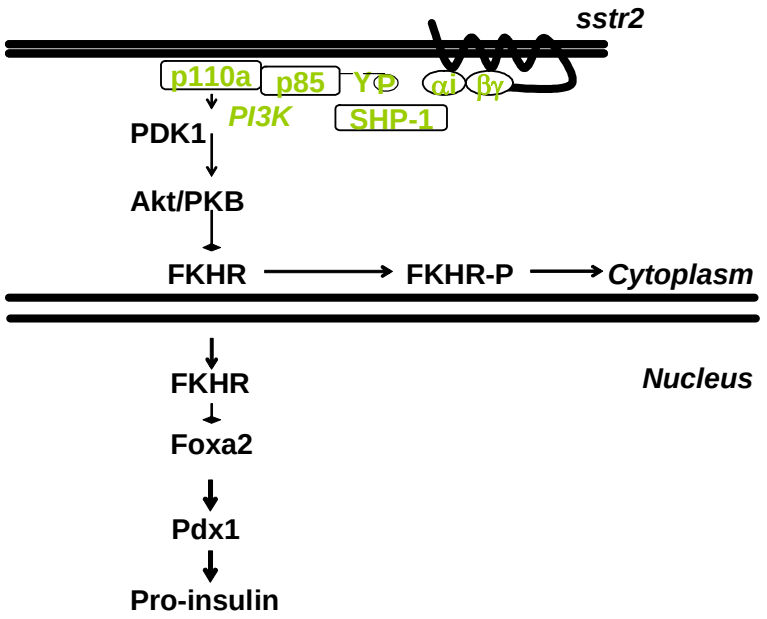


**Fig. 25.** Schematic representation of intracellular signaling followed by SSTR2 in INS-1 cells

The forkhead transcription factor Foxo1 is phosphorylated by Akt and becomes inactivated. The phosphorylated Foxo1 translocates from the nucleus into cytoplasm. Foxo1 suppresses Foxa2 (which regulates several genes responsible for insulin secretion) mediated transcription and subsequently decreases Pdx1 gene expression (downstream target molecule of PI3K pathway) and inhibits insulin secretion **(Fig. 25)**. Foxo1 competes with Foxa2 to reduce the Pdx1 promoter activity. It is known that Foxo1 inactivation leads to increase in Pdx1 expression and β-cell proliferation (Kitamura et al. 2002). In other words Foxo1 inhibits β-cell proliferation by suppressing Pdx1 gene expression.

Our data suggest that SST-14 and SSTR2 selective agonist suppress insulin secretion by inhibiting members of the PI3K pathway and subsequently Foxo1 phosphorylation.

Previous studies have demonstrated that insulin is involved in nuclear translocation of Pdx1 (Elrick et al., 2001). Insulin induces phosphorylation of Akt which activates translocation of Foxo1 to the cytoplasm. Data shows that SSTR2 inhibited total expression of pAkt and pFoxo1. The present study does not clearly demonstrate what happens at the nuclear levels. Further work needs to be done to identify the role of SSTR2 in regulation of insulin secretion via Foxo1 in INS-1 cells.

Development of Foxo1 targeting drugs opens a new pave for the treatment of diabetes which can improve β- cell proliferation. Forkhead transcription factors are actively involved in metabolism. This opens an interesting field to unveil the causes and to understanding the treatment of metabolic diseases.

Altogether the data from the present study shows that SSTR2 is a potent inhibitor of both insulin and glucagon secretion from isolated human islets and from permanent cell models of pancreatic A- and B-cells. In addition, the present study shows that by the deletion of SSTR2 gene in mice with DIO, an impaired glucose homeostasis was observed.

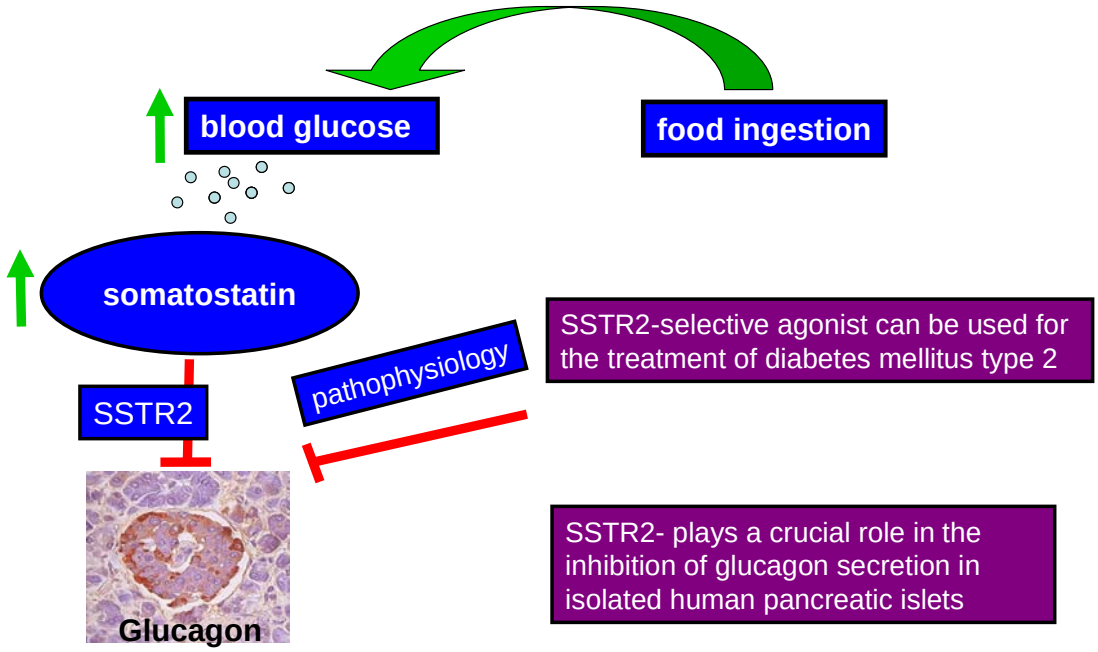


**Fig. 26.** Schematic representation of novel mechanism of glucose –dependent inhibition of glucagon secretion and potential therapeutical application.

The present study demonstrates for the first time the important link between SSTR2 and inhibition of glucagon secretion in rodents **(Fig.26)**. Since SSTR2 plays a very potent role in both insulin and glucagon secretion from human pancreas, further work needs to be done to develop highly selective agents which can selectively inhibit glucagon secretion. Unfortunately, further *in vivo* evaluation of the role of SSTR2 in regulation of glucose homeostasis is limited due to lack of clinically approved highly SSTR2-selective agonists. Octreotide is used to treat hypersecretory disorders. It shows a considerable interaction with SSTR2, SSTR5 and SSTR3. Although octreotide reduces glucagon secretion it also suppresses insulin secretion.

This study demonstrates the possible explanation for the failure of octreotide in the treatment of T2DM. On the other hand, long term treatment with octreotide causes gall stones in patients because it inhibits gall bladder contraction (Van Liessum et al., 1989)

Therefore, further work is needed to develop a highly selective glucagon inhibitor which can be used clinically to treat hyperglycemia in T2DM and/or very seldom glucagon-secreting neuroendocrine tumors.

In summary, these studies demonstrates that-

1. In humans, SST regulates both insulin and glucagon secretion mainly through SSTR2. In addition, SSTR1 and SSTR5 are also involved in the inhibition of insulin and glucagon secretion in humans.

2. Hypersecretion of glucagon is observed in diabetic patients due to breakdown of deposited glycogen content. Consistent to this, the present study shows that SSTR2 is actively involved in regulation of glucose homeostatsis in $SSTR2^{-/-}$ mice, fed with HFD. Chronic feeding with HFD made mice obese and insulin resistant which are the hallmarks of diabetes. In these $SSTR2^{-/-}$ null mice, an increase in postprandial levels of glucose and glucagon was observed, while there was a decrease in fasting levels of nonesterified fatty acids. Pancreatic islets isolated from these mice showed an impair inhibition of glucagon secretion when treated with glucose (similar situation is observed in T2DM). A postprandial decrease in hepatic glycogen and lipid content was observed in these mice. Furthermore, enzymes regulating glycogen breakdown were upregulated while enzymes stimulating glycogen synthesis were downregulated. In addition, enzymes regulating glycogenolysis and lipolysis were up-regulated in these mice. All these data clearly suggest that SSTR2 has a very important role in the regulation of glucagon secretion. Increased levels of glucagon secretion in these mice was due to increased hepatic glycogen breakdown, less lipid accumulation and also impaired regulation by glucose.

3. The intracellular signalling data for the insulin secretion in INS-1 cells shows, that SSTR2 inhibited the expression of pFoxo1 and pAkt, which play the most important role in insulin secretion. Further work is required to unveil the role of SSTR2 on nuclear translocation of forkhead transcription factors, Pdx1 and Akt.

Taken together, we summarize that our findings may prove to be potentially of clinical importance. SSTR2 can be targeted for the development of a drug which can selectively inhibit increased glucagon secretion in T2DM.