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The GLP1R agonist liraglutide reduces hyperglucagonemia induced by the SGLT2 inhibitor dapagliflozin via somatostatin release

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Summary

The newest classes of anti-diabetic agents include sodium-glucose-cotransporter-2 (SGLT2) inhibitors and glucagon-like-peptide-1-receptor (GLP1R) agonists. The SGLT2 inhibitor dapagliflozin, reduces glucotoxicity by glycosuria, but elevates glucagon secretion. The GLP1R agonist liraglutide inhibits glucagon, therefore, we hypothesize that the co-treatment of dapagliflozin with liraglutide could reduce hyperglucagonemia / hyperglycemia. Here we use five complementary models: human islet cultures; healthy mice; *db/db* mice; diet induced obese (DIO) mice; and Somatostatin receptor-2 (SSTR2) KO mice. A single administration of liraglutide and dapagliflozin in combination improves glycemia and reduces dapagliflozin-induced glucagon secretion in diabetic mice. Chronic treatment with liraglutide and dapagliflozin produces a sustainable reduction of glycemia compared to each drug alone. Moreover, liraglutide reduces dapagliflozin-induced glucagon secretion by enhancing somatostatin release, as demonstrated by SSTR2 inhibition in human islets and in mice. Collectively, these data provide mechanistic insights how intra-islet GLP1R activation is critical for the regulation of glucose homeostasis.

Keywords: Dapagliflozin, Liraglutide, GLP1R, Glucagon, Insulin, Somatostatin, Type 2 Diabetes

Introduction

Glucotoxicity is a major determinant of pancreatic islet cell demise seen in type 2 diabetic (T2D) individuals (Prentki and Nolan, 2006). In addition, postprandial hyperglucagonemia and endogenous glucose production (EGP) are also key contributors to exaggerated postprandial glucose excursions (DeFronzo et al., 1989; Dunning and Gerich, 2007; Muller et al., 1970; Rizza, 2010). How these combined abnormalities exacerbate T2D is unclear. Clearly, postprandial insulin secretion is decreased and delayed, and glucagon is not suppressed (Muller et al., 1970). Therefore, agents that can reduce glucotoxicity and postprandial hyperglucagonemia will most likely improve glycemic control. The newest classes of anti-diabetic agents include sodium-glucose-cotransporter-2 (SGLT2) inhibitors and glucagon-like-peptide-1-receptor (GLP1R) agonists. Both agents are associated with weight-loss, a decrease in A1c levels, a low-rate of hypoglycemia and decreased risk of heart failure (Bailey et al., 2010; Bajaj et al., 2017; Chen et al., 2017; Ferrannini et al., 2015; Frias et al., 2016; Frias et al., 2018; Jabbour et al., 2018; Ludvik et al., 2018; Lundkvist et al., 2017; Marso et al., 2016; Merovci et al., 2015; Zinman et al., 2015).

The primary mechanism of SGLT2 inhibitors is to inhibit renal glucose reabsorption by glycosuria, thus reducing glucotoxicity and improving insulin sensitivity and beta cell function (Abdul-Ghani et al., 2015; Merovci et al., 2015; Merovci et al., 2014; Rossetti et al., 1990). Secondary to this, they also elevate glucagon secretion and EGP (Ferrannini et al., 2014; Merovci et al., 2014), a phenomenon that was later explained at least in part, by the specific expression of SGLT2 in human pancreatic alpha cells (Bonner et al., 2015). It was later suggested that blocking SGLT2 in alpha cells partly relieves glucose suppression of glucagon secretion by allowing full-scale action potentials to develop (Pedersen et al., 2016). Recent clinical trials such as AWARD-10 and DURATION-8 have tested the efficacy SGLT2 inhibitors with GLP1R agonists for the treatment of T2D individuals, and have found that this

combination significantly improved glycemic control better than each drug alone (Frias et al., 2016; Frias et al., 2018; Ludvik et al., 2018). This is of great interest for clinical diabetology as reviewed recently by DeFronzo (DeFronzo, 2017), however, the mechanism of action of the combination on human pancreatic hormone secretion was not established. A recent study by Ørgaard and colleagues have shown that the glucagon-lowering effect of GLP-1 is mediated through the paracrine actions of somatostatin in a perfused mouse pancreas model (Orgaard and Holst, 2017), however, many aspects of GLP1 signaling in the human islet remain unresolved. This prompted us to investigate if the glucagon-lowering actions of the GLP1R agonist, liraglutide could also reduce the glucagon over-secretion induced by the SGLT2 inhibitor dapagliflozin, using human islet cultures and mouse models of diabetes. Our pre-clinical study was designed to (1) compare the efficacy of liraglutide and dapagliflozin as mono-therapies or in combination, for the long-term treatment of T2D, and (2) to provide a mechanistic insight for the combination of SGLT2 inhibitors and GLP1R agonists.

Results

A single administration of liraglutide alone or in combination with dapagliflozin suppresses glycemia and glucagon levels in diabetic mice

We first studied the effect of liraglutide alone, dapagliflozin alone or both drugs in combination in *db/db* mice. A single administration of liraglutide alone or in combination with dapagliflozin significantly reduced glycemia (Figure 1A). Liraglutide reduced plasma glucagon levels alone and in combination with dapagliflozin in fasted diabetic mice, which have elevated fasting glycemia and hyperglucagonemia (Figure 1B). The same profile for glycemia was observed after an intra-peritoneal (i.p.) injection of glucose (Figure S1A). In addition, healthy mice had normal beta-hydroxybutyrate levels compared to *db/db* mice. Dapagliflozin potently increased beta-hydroxybutyrate levels, but liraglutide had no effect (Figure 1C), despite lowering plasma glucagon levels. After an oral glucose challenge, *db/db* mice treated with dapagliflozin had significantly higher glucagon levels, which were reduced in mice treated with dapagliflozin in combination with liraglutide (Figure 1D). A similar profile for glucagon was observed after i.p. glucose administration in *db/db* mice treated with dapagliflozin alone or in combination with liraglutide (Figure S1B).

The chronic treatment with liraglutide and dapagliflozin potently reduces glycemia and glucagon levels compared to each drug alone

In order to investigate if the combination of liraglutide and dapagliflozin has an enhanced therapeutic potential for the treatment of T2D, we next studied the effect of these drugs alone or in combination in *db/db* mice over 45 days. Glycemia during fasting was not significantly lowered by liraglutide alone. Dapagliflozin decreased fasting glycemia until day 30, an effect that was not maintained. However, when liraglutide was used in combination with dapagliflozin, there was a sustainable reduction in fasting glycemia (Figure 1E). After a

glucose challenge, the combination therapy was more effective in reducing glycemia compared to each drug alone (Figure 1F). Moreover, the glucagon lowering effect of liraglutide when combined with dapagliflozin in the acute experiment was maintained in the chronic treatment (Figure 1G).

Liraglutide reduces glucagon over-secretion induced by dapagliflozin in human islets

To determine whether GLP1R activation may be directly related to its intra-islet localization, we first examined its distribution in healthy human pancreatic sections. We show that GLP1R is predominantly expressed on the membrane in human islet cells and to a lesser extent in exocrine tissue. Double-label immunofluorescence reveals that GLP1R is highly expressed on the membrane of insulin-positive beta cells, and weakly expressed in alpha cells, but moderately expressed in somatostatin-positive delta cells (Figure S2A). Of note, the GLP1R is localized on the membrane of the islet (indicated in red), whereas insulin, glucagon and somatostatin are cytoplasmic (green), hence precluding a complete yellow merged staining. Western blot analysis showed a strong signal for GLP1R protein at the expected size (≈ 62 kDa) in healthy human islets and to a lesser extent in exocrine tissue (Figure S2B). Since liraglutide reduces glucagon over-secretion induced by dapagliflozin *in vivo*, we investigated whether it was due to a direct effect on isolated islets: human islet preparations from normoglycemic donors were treated with liraglutide alone, dapagliflozin alone or both drugs in combination in the presence of 6 mM glucose for 1h, the concentration associated with maximal inhibition of glucagon release (Walker et al., 2011). Under these conditions glucagon secretion was lower compared to islets treated with 1 mM glucose. At 6mM glucose liraglutide did not further reduce glucagon secretion. Consistent with our previous results (Bonner et al., 2015), dapagliflozin treatment increased glucagon secretion at 6 mM glucose. This effect was significantly reduced by co-treatment with liraglutide (Figure 2A), without

any significant change in insulin secretion under these experimental conditions (Figure 2B).

Liraglutide inhibits glucagon secretion in a glucose-dependent manner

To further demonstrate the pharmacological relevance of our findings *in vivo*, we next studied the effect of each drug alone or in combination in healthy C57BL/6J mice. Under fasting conditions dapagliflozin and liraglutide alone or in combination reduced glycemia. However, after a glucose challenge, only the mice treated with liraglutide showed a reduction in glycemia. The effect of dapagliflozin in reducing glycemia was negligible (Figure 2C), possibly because of the time needed for glucose clearance. In healthy mice, under fasting conditions, basal glucagon levels were elevated and further potentiated by dapagliflozin. However, liraglutide alone or in combination with dapagliflozin was unable to reduce glucagon levels (Figure 2D). After a glucose challenge, where insulin is increased and glucagon is suppressed (Unger, 1971; Van Dijk et al., 1996), liraglutide alone has no additional lowering effect on plasma glucagon concentrations. However, dapagliflozin induced glucagon secretion, an effect that was reduced by the combination with liraglutide (Figure 2E), thus confirming that the action of liraglutide is glucose dependent. Moreover, we demonstrate that the glucagon-lowering action of liraglutide is more effective when glucagon levels are elevated beyond the physiological range, for example dapagliflozin-induced glucagon secretion or during hyperglucagonemia. Similar profiles for glycemia and glucagon were observed in healthy mice during fasting and after refeeding (Figure S2C-D). In healthy mice after a glucose challenge, insulin secretion was induced by a single administration of liraglutide, whereas dapagliflozin alone or in combination with liraglutide had no effect on insulin secretion (Figure 2F). Of note, mice administered dapagliflozin and liraglutide in combination increased somatostatin secretion compared to each drug alone (Figure 2G).

Blockade of SSTR2 in human islets blunts the inhibitory effect of somatostatin on glucagon secretion

Since the combination therapy showed an increase in somatostatin levels *in vivo*, we tested whether its glucagon-lowering effect is mediated via a direct action on pancreatic delta cells. Using a cross-sectional cohort we found *SSTR2* gene expression to be elevated in islets from T2D individuals compared to obese (non-diabetic) and lean individuals (Figure S3A). We also found that *GLP1R* gene expression was induced in the islets of obese non-diabetic and T2D individuals compared to healthy individuals (Figure S3B). We confirmed by confocal immunofluorescence imaging techniques that SSTR2 colocalized with insulin and glucagon in human pancreatic tissue (Figure S3C). We next demonstrated that incubation of human islets with the selective SSTR2 inhibitor CYN154806 blunted the inhibitory effect of liraglutide on dapagliflozin-induced glucagon secretion (Figure 3A). Since glucagon secretion was already decreased at 6 mM glucose, CYN154806 alone had no further effect (Figure 3B).

Liraglutide inhibits glucagon over-secretion *in vivo* via somatostatin release

We next investigated the effects of the pharmacological treatment of dapagliflozin and liraglutide in the presence of the SSTR2 inhibitor CYN154806 in DIO mice. Each drug alone or in combination reduced glycemia after a glucose challenge (Figure 4A). Liraglutide decreased the dapagliflozin-induced glucagon secretion, but when CYN154806 was co-administered with the combination therapy, it reduced the inhibitory effect of somatostatin on glucagon secretion (Figure 4B). Although, the plasma insulin levels were higher in mice treated with liraglutide, these changes were not significant (Figure 4C), most likely because somatostatin secretion was increased (Figure 4D).

Somatostatin requires SSTR2 to exert its glucagon-lowering effect

We used SSTR2 deficient mice (Zheng et al., 1997) to further demonstrate that the inhibition of glucagon secretion by liraglutide in alpha cells requires SSTR2. Firstly, we validated the specificity of SSTR2 antibody by showing the absence of its expression in the pancreatic islets of the SSTR2 KO mice compared to littermate control mice (Figure S3D). SSTR2 KO mice showed decreased glycemia after a glucose challenge compared to their littermate control mice (Figure S3E), possibly due to the mild hyperinsulinemia in these mice (Figure S3F). The acute glyceemic response to the drugs in SSTR2 KO mice (Figure S3G) was similar to that found in healthy mice. However, SSTR2 KO mice had a marked hyperglucagonemia compared to control mice, which was not reduced by liraglutide alone or the combination treatment (Figure 4E). These data confirm *in vivo* that the effect of combination of liraglutide and dapagliflozin is mediated by the action of somatostatin via SSTR2 to reduce glucagon release.

Discussion

Glucagon is a strong stimulator of hepatic glucose production. In T2D patients, glucagon secretion is not anymore suppressed after a glucose challenge (Meier et al., 2006; Muller et al., 1970) and is further exacerbated by SGLT2 inhibitors (Ferrannini et al., 2014; Martinez et al., 2018; Merovci et al., 2015). In light of these emerging issues, a drug combination that can reduce hyperglucagonemia and EGP independent of insulin therapy could be an attractive adjunct therapy for the treatment of diabetes. Our pre-clinical data demonstrate that the combination of GLP1R agonists and SGLT2 inhibitors attenuates glucagon over-secretion in diabetic mice to produce sustainable glycemic control both during fasting and after a glucose challenge in the long-term, better than each agent alone. These findings are in line with recent clinical trials in patients with T2D, which have demonstrated that, the combination of GLP1R agonists and SGLT2 inhibitors reduce A1c levels more effectively than GLP1R agonist alone (Frias et al., 2016; Goncalves and Bell, 2017; Ludvik et al., 2018). We also observed that a single administration of dapagliflozin induced circulating beta-hydroxybutrate levels, and these levels were maintained when co-administrated with liraglutide, despite reduced plasma glucagon concentrations. These data indicate that the decreased glucagon upon SGLT2 inhibition and GLP1R agonist treatment does not impede the production of ketone bodies upon prolonged fasting. The increase in ketone body metabolism after SGLT2 inhibitor treatment was shown to improve cardiac fuel utilization, thus reducing the risk of heart failure; however this is only one of a multitude of plausible mechanisms involved in the beneficial cardiac effects of SGLT2 inhibitors (Staels, 2017). Moreover, a recent study showed that the combination of GLP1R agonists and SGLT2 inhibitors reduced plasma glucagon levels in T2D patients, but EGP remained elevated (Martinez et al., 2018). These findings together suggest that factors other than glucagon secretion can participate to increase EGP and ketone body production by the liver. Indeed, ketone body production is also under

control of other hormones such as insulin, thyroid hormones and other factors such free fatty acids and catecholamines whose production are increased during prolonged fasting (Keller et al., 1989), as was the case in our overnight fasting protocol. Further studies are needed to clarify these sequential events. We also observed in healthy mice under fasting conditions in which glucagon is elevated, that liraglutide did not reduce glucagon secretion induced by dapagliflozin; thus confirming that the effect of liraglutide is glucose dependent. These findings explain the low-risk of developing hypoglycemia during treatment with these agents (Trujillo et al., 2015). Notably, we found in human islet cultures and in healthy mice *in vivo* that the glucagon-lowering effect of liraglutide was more profound when combined with dapagliflozin. Whether this is solely mediated by a direct action of GLP1R on glucagon-secreting alpha cells or whether dapagliflozin is also inducing endogenous GLP1 production from the pancreatic islets (Dunning et al., 2005; Ferrannini et al., 2014; Millar et al., 2017; Timper et al., 2016) remains unclear.

Despite the numerous studies exploring the effects of GLP1R agonists, the precise mechanism by which GLP1 reduces glucagon remains controversial. Some suggest that GLP1 could act via an intrinsic mechanism (De Marinis et al., 2010), while others propose it works via the paracrine actions of insulin (Samols et al., 1986) or somatostatin (Orgaard and Holst, 2017). Recent studies suggest a direct effect on alpha cells because the inhibition was not reversed by the insulin receptor antagonist S961 or the SSTR2 inhibitor CYN154806 (Ramracheya et al., 2018).

Somatostatin potently inhibits insulin and glucagon release from pancreatic islets via two distinct receptors (SSTR2 and SSTR5). SSTR2 is the functionally dominant somatostatin receptor expressed in both human pancreatic alpha and beta cells (Kailey et al., 2012). Therefore, in human pancreatic islets, somatostatin induced by liraglutide should exert its

inhibitory effect on glucagon and insulin secretion. Using human islet cultures, we demonstrated that liraglutide reduces glucagon secretion induced by dapagliflozin via intra-islet somatostatin release, because blockade of SSTR2 present on alpha cells blunted its inhibitory effect. In healthy subjects, the postprandial glucose rise reduces glucagon, unlike in diabetic subjects (Meier et al., 2006). In this study, we used islets isolated from normoglycemic, lean individuals, and therefore, liraglutide would not further reduce glucagon or induce insulin secretion. We believe that this experimental setting is the most appropriate to demonstrate a crosstalk between dapagliflozin, which induces glucagon secretion, and liraglutide, which enhances somatostatin secretion to suppress the glucagon induction. The proposed mechanism of action *in vitro* is summarized in the graphical abstract. However, we acknowledge that further studies on islets isolated from obese diabetic individuals would be of interest to assess the direct impact of our findings in the pathological condition of diabetes.

We also assessed the metabolic actions of dapagliflozin in combination with liraglutide in *db/db* mice and DIO diabetic mice. The combination therapy showed an improvement of glycemia and hyperglucagonemia. Although plasma insulin levels were elevated by liraglutide treatment, these values did not reach significance. The attenuation of the increase of insulin secretion by liraglutide could be due to the paracrine inhibitory action of somatostatin, which was significantly elevated in these mice. In general, clinical studies on T2D patients report an increase of insulin or c-peptide secretion in response to liraglutide treatment (Matsumoto et al., 2013; Shankar et al., 2018; Vanderheiden et al., 2016; Vilsboll et al., 2008), but a more recent study has reported the contrary (Anholm et al., 2019).

In summary, our findings demonstrate that liraglutide reduces hyperglucagonemia induced by dapagliflozin via somatostatin release. Moreover, the chronic dual-treatment of dapagliflozin and liraglutide produce a sustainable improvement in glycemia and hyperglucagonemia,

better than each agent alone, and thus provide mechanistic insights to the ongoing clinical trials with the combination of SGLT-2 inhibitors and GLP1R agonists for the treatment of diabetes. However, our study has several limitations. Since, due to limited material availability, we could not measure somatostatin secretion *in vitro* in parallel to glucagon and insulin by the same donor islet preparations, we demonstrated the action of liraglutide to reduce glucagon secretion, indirectly via blockade of the SSTR2 present on alpha cells. Moreover, the circulating forms of somatostatin, SST-14 (pancreatic) and SST-28 (intestinal) are both detected by the EIA kit used in this study. Nevertheless, we believe our findings are of interest to be further explored in future clinical studies and or clinical trials.

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Author contributions

CS and CB designed and performed most experiments, analyzed the data, and wrote the manuscript. JT, VG, EM, ND, GP, AC and AQ performed specific experiments. C.Bousquet and YM provided the SSTR2 KO mice. JKC, MD, TH and MCV participated in experimental design of specific experiments. CB, FP and BS supervised the project and edited the manuscript. All authors approved the final version of the paper.

Declaration of interest

The authors have no conflict of interest to declare.

Figure legends:

Figure 1. Acute and chronic treatment with liraglutide, dapagliflozin or the combination in *db/db* mice

Diabetic (*db/db*) mice received a single administration of vehicle (red) liraglutide (blue), dapagliflozin (green) or liraglutide and dapagliflozin in combination (purple), after 90 min mice received an oral bolus of glucose (20%). A control group of healthy C57Bl/6J mice also received vehicle and glucose (dotted black). (A) Glycemia (glycemic curve and AUC above baseline after a glucose challenge) ($n = 10-18$; overall p value: $p < 0.0001$), (B) fasting glucagon ($n = 18$; overall p value: $p < 0.0001$), (C) fasting β -hydroxybutyrate ($n = 4$; overall p value: $p < 0.0001$), specific p value **** $p < 0,0001$ and (D) glucagon after an oral glucose challenge ($n = 6$; overall p value: $p < 0.0001$). *db/db* mice were treated with vehicle, liraglutide, dapagliflozin or both in combination for 45 days and measured for (E) fasting glycemia ($n = 6$; overall p value: $p < 0.0001$), (F) glycemia ($n = 6$; overall p value: $p < 0.0001$) and (G) glucagon ($n = 6$; overall p value: $p = 0.0052$) after an i.p. injection of glucose. Specific p values: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, one-way ANOVA and Kruskal-Wallis multiple comparison for glycemia and glucagon after single drug administration and two-way ANOVA and Tukey multiple comparison test for glycemia and glucagon over 45 days.

Figure 2. Single drug administrations *in vitro* in human islets and *in vivo* in C57BL/6J healthy mice

Human islets were cultured in 1mM and 6 mM of glucose and treated with liraglutide (10 pmol/L), dapagliflozin (12 uM) or the combination. (A) Glucagon secretion (normalized to glucagon content per hour) ($n = 6$ donors; overall p value: $p < 0.0001$) and (B) insulin secretion (normalized to insulin content per hour) ($n = 10$ donors; overall p value: $p = 0.0033$). Data are expressed as means \pm s.e.m. Specific p values: $*P < 0.05$, $**P < 0.01$; $***P < 0.001$; $****P < 0.0001$, two-way analysis of variance (ANOVA) and Tukey's multiple comparison test. Healthy C57BL/6J mice were treated with vehicle (red) or liraglutide (blue) or dapagliflozin (green) or both in combination (purple). After 90 min an i.p. of glucose (20%) was administered. (C) glycemia (glycemic curve and AUC above baseline after a glucose challenge) (overall p value: $p < 0.0001$), (E) fasting glucagon (overall p value: $p < 0.0001$), (F) glucagon (overall p value: $p = 0.0011$), (G) insulin (overall p value: $p = 0.0416$) and (H) somatostatin (overall p value: $p = 0.024$) after an i.p. injection of glucose were measured. Data are expressed as means \pm s.e.m. $***P < 0.001$, one-way ANOVA and Tukey's multiple comparison test.

Figure 3. A single dose of liraglutide and dapagliflozin with and without the SSTR2 inhibitor CYN154806 in human islets

(A) Glucagon secretion (normalized to glucagon content per hour) at 6 mM glucose with or without liraglutide (10 pmol/L), dapagliflozin (12 uM) and CYN154806 (100 nM); $n = 4$ donors (overall p value: $p < 0.0001$) and (B) with and without CYN154806. Data are expressed as means \pm s.e.m. . Specific p values: $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$, two-way analysis of variance (ANOVA) and Tukey's multiple comparison test.

Figure 4. Acute treatments of liraglutide and dapagliflozin with and without the SSTR2 inhibitor CYN154806 in SSTR2 KO mice and DIO mice

(A) Glycemia (glycemic curve and AUC above baseline after a glucose challenge) ($n = 10$; overall p value: $p < 0.0001$) for DIO mice treated with vehicle (red), liraglutide (blue), dapagliflozin (green), both in combination (purple) and in the presence of SSTR2 inhibitor CYN154806 (orange), (B) glucagon ($n = 10$; overall p value: $p < 0.0001$), (C) insulin ($n = 6$; overall p value: $p = 0.0004$) and (D) somatostatin ($n = 6$; overall p value: $p = 0.0001$), after an i.p. injection of glucose (20%) were measured. (E) Glucagon plasma levels after an i.p. of glucose (20%) in WT mice (white) or SSTR2 KO mice treated with vehicle (red), liraglutide (blue), dapagliflozin (green) or the combination (purple) ($n = 8$; overall p value: $p < 0.0001$). Specific p values: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$, one-way ANOVA and Tukey's multiple comparison test were used.

STAR Methods

LEAD CONTACT AND MATERIALS AVAILABILITY

This study did not generate new unique reagents. Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Caroline Bonner (caroline.bonner@univ-lille.fr).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

All experimental procedures were performed by at least two investigators, and some but not all experiments were performed in a blinded fashion. No data points were excluded from analyses in mice or human islet studies. Human pancreatic tissue and islets was harvested from brain-dead adult human donors in the context of the traceability requirements for our clinical islet transplantation program (clinicaltrials.gov, NCT01123187, NCT00446264, NCT01148680). Age/sex and metabolic phenotype were provided for all donors (Table S2).

Animals were not randomized for vehicle, dapagliflozin alone, liraglutide alone or for the combination of dapagliflozin and liraglutide treatments for the acute experiments, but were randomized for the chronic experiments. Sample sizes were chosen with adequate power based on the literature. In vivo experiments were conducted independently with at least six mice per group, and repeated at least two times, when applicable, except for the chronic treatments which was performed once over 45 days. The experimental design was approved in agreement with French regulations, our Institutional Ethical Committee of the University of Lille and the Centre Hospitalier Régional Universitaire de Lille.

In vivo animal studies

Control male C57BL/6J mice (aged 10-12 weeks) and *db/db* male mice (aged 10-12 weeks) were purchased from Elevage Janvier laboratories (Le Genest-Saint-Isle, France), were

housed at 22±2°C with 12:12 h light/dark cycle. We chose C57BL/KsJ-*db/db* mice at 12 weeks of age because they exhibit many of the early metabolic disturbances of human T2D, including obesity, hyperglycemia, early hyperinsulinemia, hyperglucagonemia and renal pathology (Berglund et al., 1978; Hummel et al., 1966). SSTR2 KO mice were obtained from L'Equipe labellisee Ligue Contre le Cancer Toulouse, France. All animals had free access to standard chow diet (Safe - A04 16% Protein Rodent Diet) and water. A group of male C57BL/6J mice had free access to high fat diet (HFD) (Research diet - D12492i, 60% fat; Research Diet Brogaarden, Denmark) for 3 months. All animal procedures were conducted in accordance with the institutional guidelines approved by the institutional Animal Care and Ethical Use Committee of the University of Lille, France. Control healthy mice, *db/db* mice and mice receiving a HFD were fasted and split into 4 groups and treated acutely with the different drugs (Table S1). Glycemia was monitored before and after drug administration (time 0, 30, 60 and 90 min). After 90 min from drug administration all mice received an i.p. injection of glucose (2g/kg) and glycemia was measured after 30 or 60 min. An i.p. route was chosen to avoid the stimulation of endogenous GLP1 and to bypass the effect of liraglutide on reducing food intake. Blood samples were collected and pancreatic hormones were measured during fasting and after a glucose challenge. For the chronic experiment *db/db* mice were split into 4 groups as above and received the drug treatments for 45 days (dapagliflozin 5 mg/Kg and liraglutide 0,15 mg/Kg). Glycemia was monitored during fasting and after a glucose challenge at days 10, 20, 30 and 45. The same experimental protocol for acute treatment was followed and blood samples were collected.

METHOD DETAILS

Hormone measurements

Glucagon concentrations were measured using the Glucagon Quantikine ELISA Kit (R&D Systems Europe, Lille, France) according to the manufacturer's instructions. Insulin

concentrations were measured using the mouse Insulin Elisa kit (Merckodia AB; Uppsala Sweden), and Somatostatin was measured using the Enzyme Immunoassay kit for Somatostatin (Phoenix Pharmaceuticals, Inc, Aachen Germany).

Beta-hydroxybutrate measurements

Beta-hydroxybutrate concentrations were measured using the beta-hydroxybutrate colorimetric kit (Abcam ab83390, France) according to the manufacturer's guidelines.

Tissue processing and sectioning

Human pancreatic tissue was harvested from brain-dead adult human donors in the context of the traceability requirements for our clinical islet transplantation program (clinicaltrials.gov, NCT01123187, NCT00446264, NCT01148680), fixed in 4% (w/v) paraformaldehyde and embedded in paraffin. Sections (10 µm) were cut on a Leica RM 2165 microtome (Leica, Copenhagen, Denmark). The experimental design and protocols used in this study was approved in agreement with French regulations, our Institutional Ethical Committee of the University of Lille and the Centre Hospitalier Régional Universitaire de Lille.

Immunostaining techniques

To study the expression of GLP1R proteins in the pancreas, two antibodies were tested. The first antibody was sourced from Abcam, rabbit polyclonal GLP1R (1:100; Abcam Cat# ab39072, RRID: AB_880213) and was found to be non-specific. The second was a monoclonal GLP1R hybridoma (undiluted; DSHB Cat# Mab 3F52, RRID: AB_2618100). The specificity of the anti-sera for GLP1R has been validated previously by Waser and colleagues (Waser et al., 2015). The specific expression of Somatostatin Receptor 2

(SSTR2) in the pancreas was determined by using mouse anti- SSTR2 (1:100; Santa Cruz Biotechnology Cat# sc-365502, RRID: AB_10859216). To study the specific localization of GLP1R and SSTR2 in the pancreas, we performed dual immunofluorescence staining analysis with a mixture of antibodies against islet specific hormones, which included rabbit polyclonal anti-glucagon (1:800; Abcam Cat# ab8055, RRID: AB_2247265), rabbit polyclonal anti-insulin (1:800; Abcam Cat# ab63820, RRID: AB_1925116), rabbit polyclonal anti-somatostatin (1:800; Dako Cat# A0566, RRID: AB_2688022) for co-localization studies. For single-label immunohistochemistry DAB (Diaminobenzidine) or immunofluorescence staining we followed the exact protocol by Waser and colleagues (Waser et al., 2015). In brief, paraffin sections from the human pancreas, kidney and intestine were dewaxed and rehydrated to H₂O. Sections were microwave treated for 8 min at 900W and 30 min at 360W in Tris-EDTA buffer, pH 9.0, were allowed to cool for 15 min, rinsed in Tris-buffered saline (TBS), pH 7.5, and blocked in 3% H₂O₂ with 0.15M NaN₃. Slides were incubated overnight with the primary GLP1R monoclonal mouse antibody 7 mg/ml MAb 3F5 diluted in antibody dilution buffer (S0809 Dako, Courtabouef, France). The following day, the slides were washed in 5 times in TBS for 5 mins, followed by Flex-Linker (Dako) for 20 minutes. The slides were again washed before the addition of Flex/HRP for 15 minutes. Another wash was performed, and the slides were developed with DAB (Dako). For immunofluorescence co-staining experiments, secondary antibodies used include Alexa Fluor 488 or 594 nm, anti-rabbit, or anti-mouse IgG (Thermo Fisher Scientific Cat# A-21206, RRID: AB_2535792, Cat# A-11001, RRID: AB_2534069, Cat# A-11012, RRID: AB_2534079, and Cat# A-11032, RRID: AB_2534091), and DAPI (used as nuclei counterstain) (Vector Laboratories Cat# H-1200, RRID: AB_2336790). Images were taken with a Nikon ECLIPSE Ti microscope using NIS ELEMENTS AR 3.2 software (Nikon, Champigny-sur-Marne, France). No immunolabeling was observed when the primary or

secondary antibodies were omitted.

Western blot analysis

Human islets were harvested in a lysis buffer containing 40 mM HEPES (pH 7.5), 120 mM NaCl, 1 mM EDTA, 10 mM pyrophosphate, 10 mM glycerophosphate, 50 mM NaF, 1.5 mM sodium orthovanadate, 10 μ M okadaic acid, 10 μ g/ml aprotinin and 10 μ g/ml leupeptin. After lysis, insoluble material was removed by centrifugation at 12,000 r.p.m. for 10 min at 4 °C. Protein concentration was determined using the Bradford protein assay (Bio-Rad, Marnes-la-Coquette, France). Samples were supplemented with 2-mercaptoethanol (Sigma-Aldrich, St Louis, USA), and denatured at 85°C for 10 min. An equal amount of protein (30 μ g) was separated with 12% SDS-PAGE and blotted to nitrocellulose membranes (Protean BA 85, Schleicher & Schuell, Dassel, Germany). The membranes were blocked with 5% nonfat dry milk in PBS containing 0.1% (v/v) Tween 20 for 60 min at 22 °C. The mouse monoclonal antibody for GLP1R (DSHB Cat# Mab 3F52, RRID: AB_2618100) was diluted in 5% milk to a final concentration of 1,78 μ g/mL (Waser et al., 2015), and incubated overnight at 4 °C. The mouse anti-SSTR2 (Santa Cruz Biotechnology Cat# sc-365502, RRID: AB_10859216) was diluted 1:1000 and incubated overnight. The membranes were washed in Tris-buffered saline containing 0.05% Tween 20. The anti-mouse HRP-conjugated antibody was diluted 1:1000 in 5% milk and incubated for 60 min at room temperature. The membranes were washed 3 times for 15 min in Tris-buffered saline containing 0.1% Tween 20 and blots were developed with ECL Plus, Amersham (GE Healthcare Life Science, Velizy-Villacoublay, France).

Human islet isolation and culture

For pancreatic hormone secretion experiments in response to glucose and drug treatments,

human islets were cultured from normoglycemic individuals with a BMI < 30 and > 90% viability and > 80% purity (endocrine versus exocrine tissue) in glucose-free medium (Gibco, Life Technologies, Paris, France) supplemented with 0.625% HSA, 1% P/S and 5 mM glucose. For glucose responsive experiments, human islets were exposed to 1 mM or 6 mM glucose in the presence or absence of 12 μ M of dapagliflozin alone (Bonner et al., 2015) or (10 pmol/L) of liraglutide alone (Shigeto et al., 2015) or in combination. Using static incubation techniques, drug treatment was performed for 1h incubation and hormone secretion was normalized to intra-islet protein (hormone) content. Each condition was performed in quadruple from each donor islet preparation and hormone secretion was normalized to % of intracellular content. Dapagliflozin was obtained from AstraZeneca (Mölndal, Sweden), liraglutide (stylo Victoza) was purchased from Novo nordisk® (Bagsvaerd, Denmark) and SSTR2 inhibitor CYN154806 was obtained from (Sigma Aldrich, Paris, France).

Real-time quantitative RT-PCR (qPCR)

Human islets were isolated from pancreata harvested from lean, obese and T2DM adult brain-dead individuals as described previously (Kerr-Conte et al., 2010). As recently suggested by American Diabetes Association and European Association for the Study of Diabetes (Hart and Powers, 2019), we provide a standardized table titled key characteristics of human islets and their use Table S2.

Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Courtabœuf, France). First-strand cDNA synthesis was performed using 0,5 μ g total RNA as a template and Superscript IV reverse transcriptase (Life Technologies, Saint Aubin, France) primed with 50 pmol of random hexamers (New England BioLabs, Ipswich, MA, USA). Quantitative real-time PCR was performed using the Bio-Rad MyiQ Single-Color Real-Time PCR Detection System and

the Bio-Rad iQ SYBR Green Supermix (Bio-Rad Laboratories, CA, USA) as previously described (Bonner et al., 2015). Gene expression was normalized to the housekeeping gene RPL27, in agreement with a previously described panel of house-keeping genes consistently used for this normalization (de Jonge et al., 2007). The primers for genes *GLPIR* and *SSTR2* were designed with the website Primer3; the final concentration used for all the primers was 500 nM (250 forward + 250 reverse). The cDNA was diluted 1:10 and 1uL was used in 10uL final volume for each well. The reaction was: 95°C for 3 min (priming) followed by 40 cycles, 1 cycle at 95°C for 10 sec (denaturation) and 60°C for 30 sec (annealing). A melt curve analysis was performed for quality of primers and qPCR reaction.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical Analysis

Data are expressed as means \pm s.e.m. and two-way ANOVA, with appropriate corrections such as Tukey's and Bonferroni's post hoc tests were used for multiple comparisons. For the in vivo experiments, the Shapiro-Wilk normality test was performed to test if values had a Gaussian distribution and according to this, results between groups were analyzed using ordinary one-way ANOVA with Tukey's multiple comparisons test or non-parametric Kruskal-Wallis analysis with Dunn's multiple comparison test. Statistical analyses were performed using GraphPad PRISM 7.0 (GraphPad Software, La Jolla, California, USA). Differences were considered significant at $p < 0.05$.

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Dr Caroline Bonner (caroline.bonner@univ-lille.fr).

DATA AND CODE AVAILABILITY

This study did not generate/analyze datasets/code.

Table S2. Key characteristics of human islets and their use. Related to Figure 2 and 3 and Figure S2 and S3.

* qPCR; \$ immunofluorescence; ** Western blot and # in vitro studies.

GRAPHICAL ABSTRACT

(1) SGLT2 inhibition by dapagliflozin promotes glucagon secretion by the pancreatic alpha cells
(2) GLP1R activation by liraglutide induces somatostatin secretion in delta cells, without any effect on insulin secretion in beta cells. The role of GLP1R on alpha cells is unknown. (3) Somatostatin acts on SSTR2 present on pancreatic alpha cells to attenuate glucagon secretion induced by dapagliflozin (4) Selective inhibition of SSTR2 in alpha cells by the specific inhibitor CYN154806 reversed the glucagon lowering effect of somatostatin.

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Figure 1

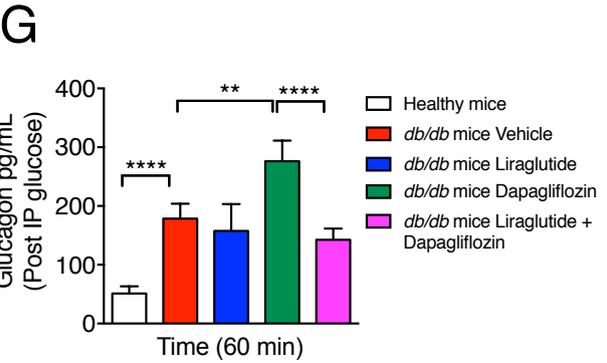
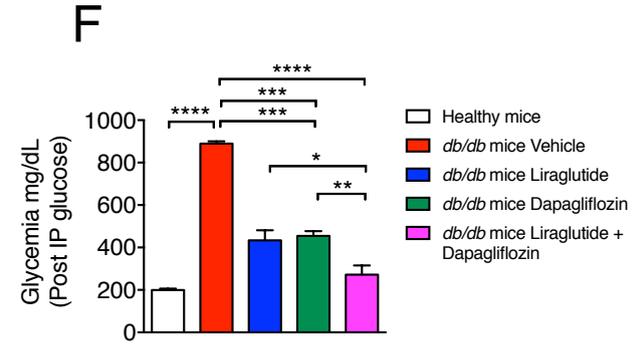
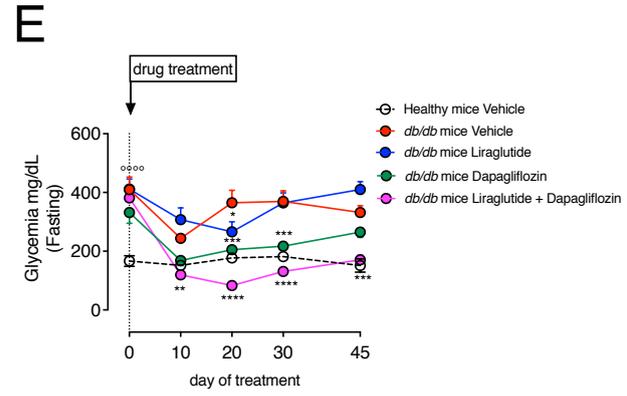
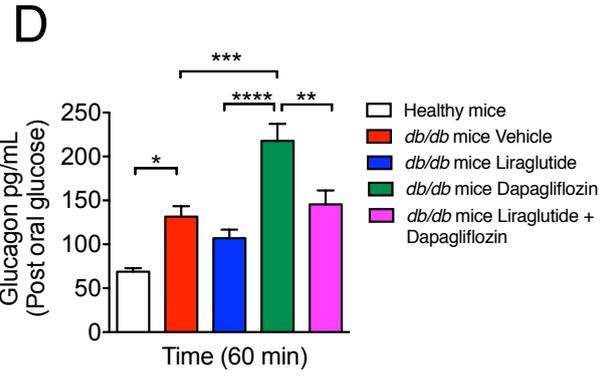
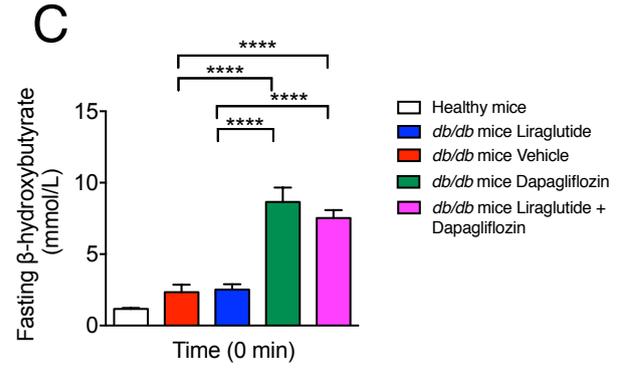
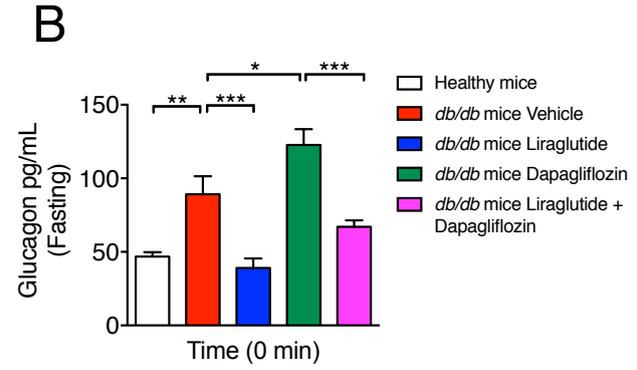
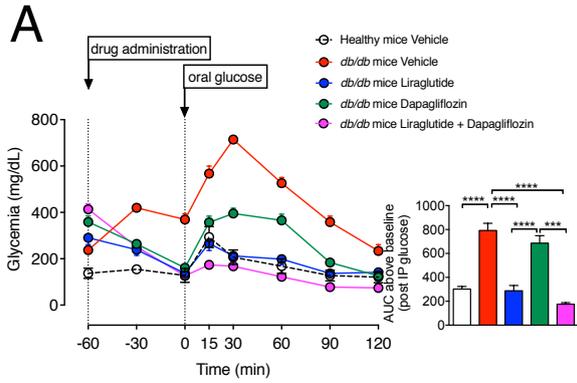
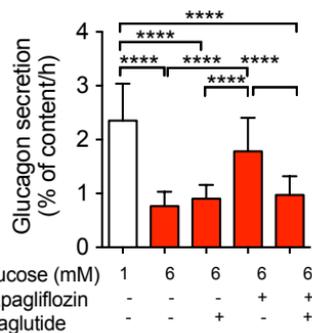
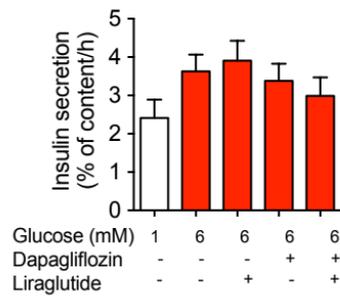


Figure 2

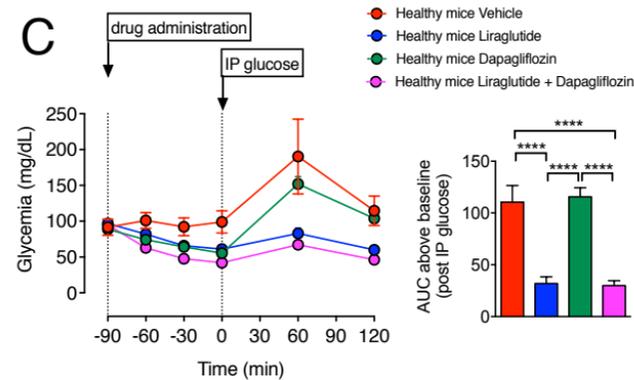
A



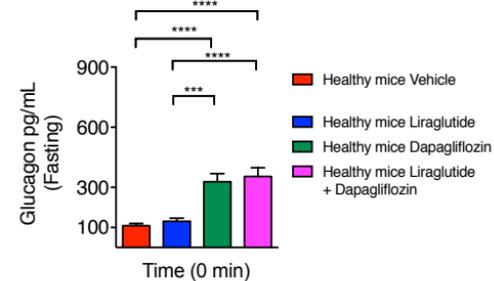
B



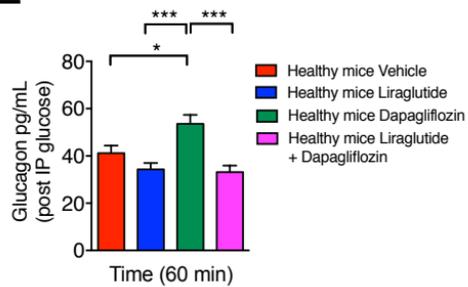
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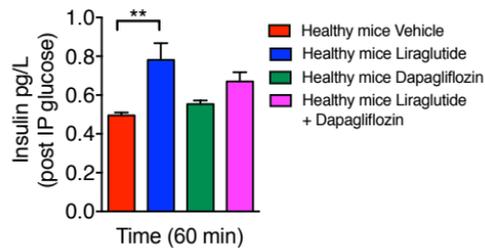
D



E



F



G

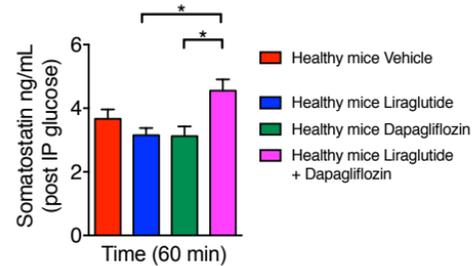
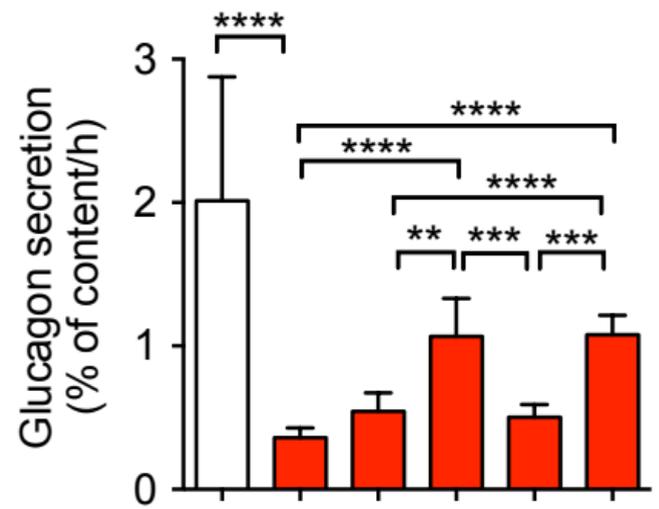


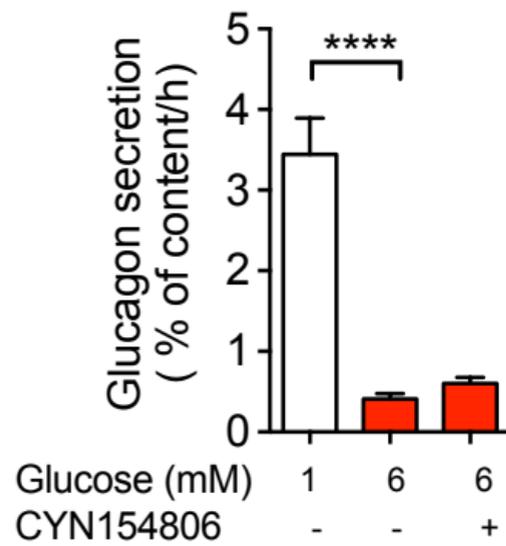
Figure 3

A



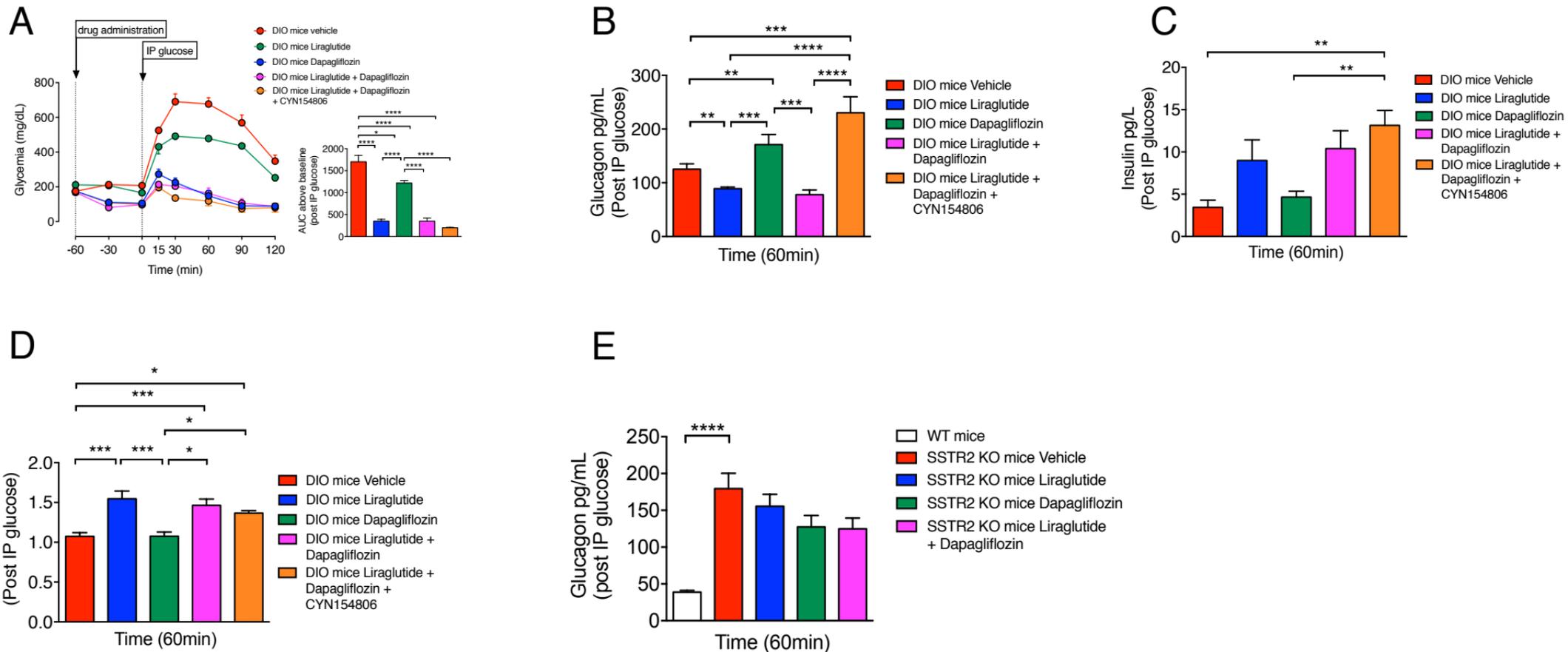
Glucose (mM)	1	6	6	6	6	6
Dapagliflozin	-	-	-	+	+	+
Liraglutide	-	-	+	-	+	+
CYN154806	-	-	-	-	-	+

B



Glucose (mM)	1	6	6
CYN154806	-	-	+

Figure 4



MECHANISM OF ACTION in HUMAN ISLETS in NORMOGLYCEMIC CONDITIONS

