Original Article

MOLECULAR METABOLISM

SGLT2 is not expressed in pancreatic α- and β-cells, and its inhibition does not directly affect glucagon and insulin secretion in rodents and humans

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ABSTRACT

Objective: Sodium-glucose cotransporter 2 (SGLT2) inhibitors (SGLT2i), or gliflozins, are anti-diabetic drugs that lower glycemia by promoting glucosuria, but they also stimulate endogenous glucose and ketone body production. The likely causes of these metabolic responses are increased blood glucagon levels, and decreased blood insulin levels, but the mechanisms involved are hotly debated. This study verified whether or not SGLT2i affect glucagon and insulin secretion by a direct action on islet cells in three species, using multiple approaches.

Methods: We tested the *in vivo* effects of two selective SGLT2i (dapagliflozin, empagliflozin) and a SGLT1/2i (sotagliflozin) on various biological parameters (glucosuria, glycemia, glucagonemia, insulinemia) in mice. mRNA expression of SGLT2 and other glucose transporters was assessed in rat, mouse, and human FACS-purified α- and β-cells, and by analysis of two human islet cell transcriptomic datasets. Immunodetection of SGLT2 in pancreatic tissues was performed with a validated antibody. The effects of dapagliflozin, empagliflozin, and sotagliflozin on glucagon and insulin secretion were assessed using isolated rat, mouse and human islets and the *in situ* perfused mouse pancreas. Finally, we tested the long-term effect of SGLT2i on glucagon gene expression.

Results: SGLT2 inhibition in mice increased the plasma glucagon/insulin ratio in the fasted state, an effect correlated with a decline in glycemia. Gene expression analyses and immunodetections showed no SGLT2 mRNA or protein expression in rodent and human islet cells, but moderate SGLT1 mRNA expression in human α-cells. However, functional experiments on rat, mouse, and human (29 donors) islets and the *in situ* perfused mouse pancreas did not identify any direct effect of dapagliflozin, empagliflozin or sotagliflozin on glucagon and insulin secretion. SGLT2i did not affect glucagon gene expression in rat and human islets.

Conclusions: The data indicate that the SGLT2i-induced increase of the plasma glucagon/insulin ratio *in vivo* does not result from a direct action of the gliflozins on islet cells.

Keywords Gliflozins; SGLT2 inhibitor; Glucagon; Insulin; Diabetes

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Abbreviations: αMG, α-methyl-D-glucopyranoside; BW, body weight; C_{max}, maximum serum concentration; EGP, endogenous glucose production; FACS, fluorescence-activated cell sorting; G, glucose; IC_{50}, half maximal inhibitory concentration; SGLT, sodium-glucose cotransporter; SGLT2i, sodium-glucose cotransporter 2 inhibitors; TPM, transcripts per million

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1. INTRODUCTION

SGLT2 inhibitors (SGLT2i), called gliflozins, decrease glycemia by blocking glucose reabsorption by the kidney, thus promoting glucosuria. They are extensively used to treat diabetes and exert other beneficial effects such as improvement of insulin sensitivity and β-cell function, cardiorenal protection, and weight loss [1—7]. SGLT2i-induced glucosuria is associated with metabolic responses, including an increase in lipolysis, endogenous glucose, and ketone body production [3,8,9]. The paradoxical increase in endogenous glucose production (EGP) is a concern, as it diminishes the efficacy of glucosuria-stimulating therapy [3]. An additional drawback of SGLT2i is the increased incidence of ketoacidosis and the emergence of rare euglycemic diabetic ketoacidosis [10—14]. The rise in ketone body levels results from their increased production rather than decreased renal clearance [15]. The potential causes of these metabolic responses are the SGLT2i-mediated increase in blood glucagon levels and decrease in blood insulin levels [3,8,10], but the mechanisms by which gliflozins increase glucagonemia are highly contested [7,16—18]. Some studies have suggested that gliflozins act directly on α-cells that express SGLT2 [19—22]. Others suggested that α-cells express SGLT1 but not SGLT2 and that gliflozins that do not display an exclusive selectivity for SGLT2 stimulate glucagon release by inhibiting SGLT1 [23,24]. An indirect control of glucagon release by gliflozins via δ-cells has also been proposed [25]. However, these hypotheses are contested by studies suggesting no SGLT2 expression in α-cells and no effect of gliflozins on glucagon release [17,26] or an inhibition of glucagon release by gliflozins [22,27]. As glucagon and somatostatin strongly influence β-cells, gliflozins might indirectly control insulin secretion, but several reports do not support this mechanism [11,14,19,26,28].

In the present study, we tested the possibility that SGLT2i affect glucagon and insulin secretion via a direct action on islet cells. To identify potential interspecies differences that might explain the controversies, this study was conducted on three species: rats, mice, and humans. To increase the strength of our analysis, we used multiple approaches. 1) We studied mRNA expression of SGLT2 and other glucose transporters in fluorescence-activated cell sorting-(FACS-)purified α- and β-cells. 2) We analyzed two human islet cell transcriptomic datasets. 3) We performed immunodetections of SGLT2 in pancreatic tissues with a carefully validated antibody. 4) We tested the effects of dapagliflozin, empagliflozin (two SGLT2i), and sotagliflozin (a dual SGLT1/2i) on glucagon and insulin secretion from isolated rat, mouse, and human islets and from the *in situ* perfused mouse pancreas. 5) We verified the effects of the gliflozins *in vivo* in mice. 6) Finally, we tested the long-term effect of SGLT2i on glucagon gene expression.

2. METHODS

2.1. Study approval

The experiments were approved by the committees for animal welfare (2014/UCL/MD/016 and 2018/UCL/MD/18) and human islets (2017/12JUL/369) at the Université Catholique de Louvain and followed the regulatory conditions of Boehringer Ingelheim's corporate policy in accordance with German legislation.

2.2. Models and tissue preparation

2.2.1. Rodent strains and islet preparation

Wistar-Han rats and C57BL/6N mice (6—12 months) were used for all experiments, except for gene expression, which was conducted using Glu-Venus [29] and RIPYY mice [30]. Islets were isolated by collagenase and cultured overnight in RPMI 1640 medium containing 11 mM (rat) or 7 mM (mouse) glucose and 10% FBS.

2.2.2. Human islets

The origin and characteristics of the human islet preparations are listed in Supplementary Table S1. After shipment, the islets were cultured for 2—17 days (mean: 5.5 d; median: 5 d) in RPMI 1640 medium containing 5 mM glucose and 10% FBS or PIM medium (Prodo Labs).

2.3. Fluorescence-activated cell sorting and gene expression measurements

2.3.1. FACS

Dispersed islet cells were FACS-sorted using methods adapted to the different species (Supplementary Figure S1).

2.3.2. cDNA preparation

RNA was extracted using Dynabead-oligo dT or TriPure and reverse transcribed into cDNA.

2.3.3. qPCR

TaqMan probes and SYBR Green were used. See Supplementary Table S2 for probe sets and primers. Changes in gene mRNA levels normalized to those of reference genes (*ACTB*, *Gapdh*, *TBP*, or *RPLP0*) are shown as $2^{-\Delta Ct}$.

2.4. Single-cell RNA-Seq analysis from public data

We downloaded two pre-processed single-cell datasets [31,32] from ArrayExpress (E-MTAB-5061) and the EBI Single-Cell Expression Atlas (E-GEOD-81547) (analysis pipeline details in the respective entries). Apart from the TPM matrix, we used inferred cell type annotation provided with the respective entries to visualize the gene expression per cell type.

2.5. ^{14}C-αMG uptake experiments and Western blotting analysis of SGLT2

^{14}C-αMG uptake experiments were conducted as previously described [33]. The anti-SGLT2 antibody was characterized by Western blotting analysis using total membrane extracts of lysates from human islets and kidneys, and HEK293 cells expressing SGLT2 or not. Western blotting analysis and PNGaseF treatment were performed as previously described [34].

2.6. Immunodetection of SGLT2 and islet hormones

Pancreatic and kidney tissues of human, mouse, and rat origin were fixed in 4% paraformaldehyde prior to paraffin embedding. Three-μm thick sections were processed for multiplex immunofluorescence. Briefly, sections were sequentially incubated for 30 min at room temperature with primary antibodies (Supplementary Table S2) and then for 30 min at room temperature with an Opal Polymer Anti-Mouse and Rabbit HRP Kit (PerkinElmer). Immunofluorescent signal was visualized using the OPAL TSA dye 520, 570, 650 and 690.

2.7. *In vivo* experiments

Drugs (dapagliflozin, empagliflozin, and sotagliflozin, 1—10 mg/kg BW) or vehicle (DMSO) were administered by oral gavage to mice either once or one dose during 3 consecutive days. ELISA kits were used to assay plasma glucagon (Mercodia) and insulin (Crystal Chem).

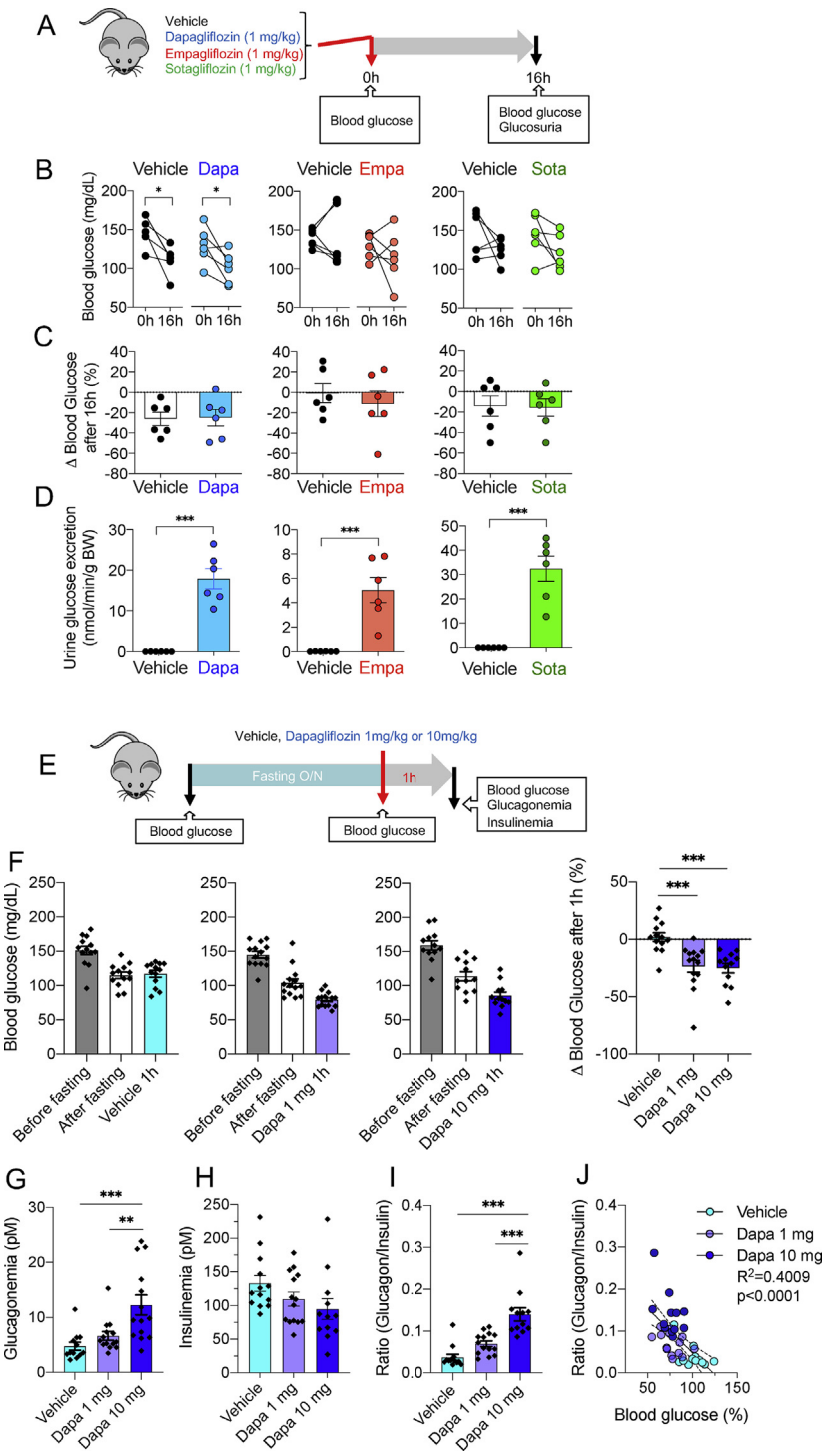

Figure 1: Gliflozins increase glucosuria and induce an increase in the glucagonemia/insulinemia ratio that correlates with a decline in glycemia in fasted mice. (*A-D*) Gliflozins efficiently increase glucosuria. (*A*) Schematic design of gliflozin treatment. C57BL/6N male mice (n = 6/group) housed in metabolic cages received by oral gavage vehicle (0.1% DMSO), dapagliflozin (Dapa), empagliflozin (Empa), or sotagliflozin (Sota) (1 mg/kg body weight [BW] for each gliflozin). Blood glucose (*B*) was measured right before oral gavage and 16 h later. Δ blood glucose between these two time points was calculated as the percentage change (*C*). Glucosuria (*D*) was measured in urine collected during this 16-h period. Graphs in panels *C-D* show individual data and means ± SE. *$p < 0.05$ and ***$p < 0.001$, paired t-test for comparison between 0 h and 16 h and unpaired t-test for comparison between vehicle and treatment. (*E*) Schematic design of dapagliflozin treatment. C57BL/6N male mice (n = 12–14/group) housed in regular cages were fasted overnight for 16 h and received by oral gavage, right after fasting, vehicle (0.6–1% DMSO) or dapagliflozin 1 or 10 mg/kg BW. Blood glucose (*F*) was measured before fasting, after fasting right before vehicle or dapagliflozin administration, and 1 h later. Δ blood glucose calculated as the percentage change before and 1 h after oral gavage is illustrated in the right panel. Glucagonemia (*G*), insulinemia (*H*), and the glucagonemia/insulinemia ratio (*I*) were measured 1 h after oral gavage. (*J*) Correlation between the changes in blood glucose (percentage taken from the right panel in *F*) and the glucagonemia/insulinemia ratio (taken from *I*). $R^2 = 0.4009$. Graphs in panels *F–I* show individual data and means ± SE. **$p < 0.01$ and ***$p < 0.001$ for comparisons between treatments, ordinary one-way ANOVA followed by Tukey's test for multiple comparisons.

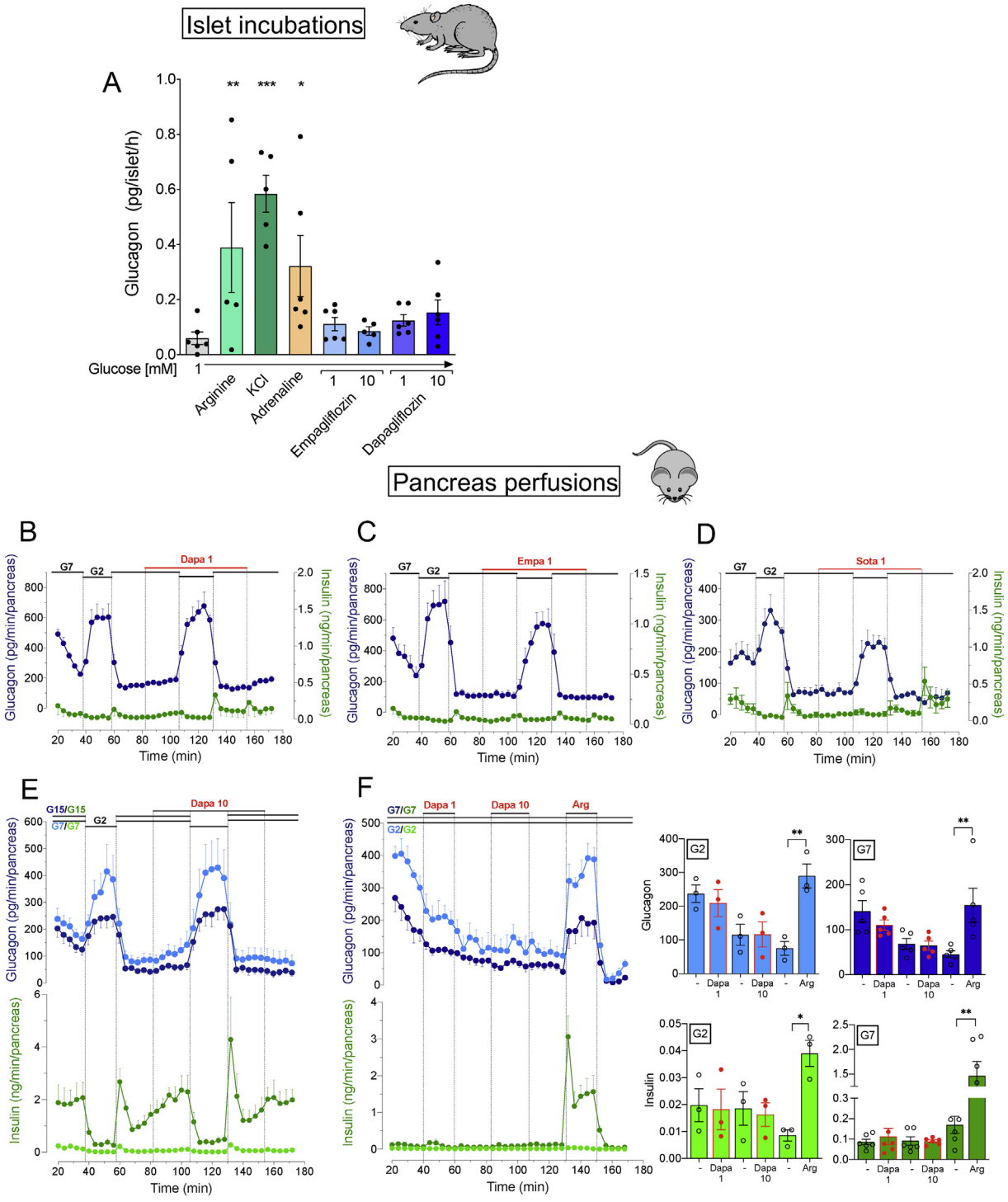

Figure 2: Gliflozins do not directly affect glucagon and insulin secretion in rodents. (*A*) Islets from Wistar-Han rats (10 islets/100 μl medium) were incubated for 1 h in a medium containing 1 mM glucose and various test agents including arginine (10 mM), KCl (60 mM), adrenaline (10 μM), empagliflozin (1 or 10 μM), or dapagliflozin (1 or 10 μM). Values are means ± SE for 5–6 batches of islets. *p < 0.05, **p < 0.01, and ***p < 0.001 for comparisons between treatments and control (G1) , ordinary one-way ANOVA followed by Fisher's LSD test for multiple comparison. The trend for glucagon secretion increase induced by 10 μM dapagliflozin was not significant (p = 0.504) (*B–F*) Pancreas from C57BL/6N mice were perfused *in situ* with a solution containing a 2 mM mixture of various amino acids present at the following concentrations, which mimics physiological concentrations (in mM): 0.4 alanine, 0.5 glutamine, 0.2 lysine, 0.25 glycine, 0.15 leucine, 0.25 valine, 0.15 threonine, and 0.1 serine. The glucose (G) concentration was changed between 7 or 15 and 2 mM, and dapagliflozin (Dapa, 1 or 10 μM), empagliflozin (Empa, 1 μM), sotagliflozin (Sota, 1 μM), and arginine (Arg, 10 mM) were applied when indicated. Traces are means ± SE of 3–8 experiments with different mice. Graphs on the right of *F* are scatter-dot plots with bar graphs showing individual data and means ± SE of the average glucagon and insulin secretions calculated from the experiments shown on the left. Because glucagon secretion displayed important spontaneous time-dependent changes, the mean secretion in the presence of a test agent (dapagliflozin or arginine) was compared to the mean secretion integrated over the control period before and after the application of the test agent (G7 or G2 vs G7 or G2 + dapagliflozin 1 μM: mean of 20–36 and 60–80 min vs mean of 40–56 min; G7 or G2 vs G7 or G2 + dapagliflozin 10 μM: mean of 60–80 and 108–128 min vs mean of 84–104 min; and G7 and G2 vs G7 or G2 + arginine 10 mM: mean of 108–128 and 156–168 min vs mean of 132–148 min). *p < 0.05, and **p < 0.01, paired t-test for comparison between different conditions.

2.8. Secretion experiments

2.8.1. Incubation experiments

These experiments were performed with rat and human islets (10 islets/100 μL medium). The medium contained (in mM): 137 NaCl, 5.4 KCl, 1.3 $CaCl_2$, 0.81 $MgSO_4$, 0.34 NaH_2PO_4, 0.44 KH_2HPO_4 + 1 mg/mL BSA, and was at pH 7.4. Islets were maintained for 30 min in a medium containing 25 (rat) or 11.1 mM (human) glucose before being transferred in a medium containing 1 mM glucose and the respective treatments. One hour later, glucagon was determined using a Fluorescent EIA Kit (Phoenix Pharmaceuticals).

2.8.2. Dynamic secretion experiments

Experiments on perifused mouse and human islets and perfused mouse pancreas were performed as previously described [35]. The medium contained (in mM): 124 NaCl, 4.8 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 20 $NaHCO_3$ + 1 mg/mL BSA, and was at pH 7.4. Except where otherwise indicated, it was supplemented with a 6 mM mixture (for the perifused islets) or 2 mM mixture (for the perfused pancreas) of amino acids (see figure legends). Insulin (home-made assay) and glucagon (Merck Millipore) were measured by radioimmunoassays.

2.9. Statistical methods

Statistical significance of differences between means was evaluated by paired t-tests or one-way ANOVA followed by Tukey's or Fisher's LSD test as described in the figure legends and results.

3. RESULTS

3.1. *In vivo* experiments

To verify the efficiency of the gliflozins used, dapagliflozin, empagliflozin, sotagliflozin (1 mg/kg BW), or vehicle (DMSO) were administered by oral gavage in mice maintained for 16 h in metabolic cages and their urine was collected (Figure 1A). Glycemia tended to decrease during this 16-h period but gliflozins did not exacerbate this drop (Figure 1B–C). As expected, all of the gliflozins strongly increased glucosuria, whereas the vehicle had no effect (Figure 1D).

In another series of experiments, we tested the effect of dapagliflozin (1–10 mg/kg BW) on glucagonemia and insulinemia. Mice were fasted for 16 h (a condition where glucagonemia is increased) before receiving a single dose of either vehicle or gliflozin (Figure 1E) and their blood was collected 1 h later. As expected, fasting significantly decreased glycemia in all groups (Figure 1F). Dapagliflozin induced a further decrease in glycemia 1 h later and significantly increased glucagonemia and the glucagon/insulin ratio (without affecting insulinemia) (Figure 1F–J). The glucagon/insulin ratio significantly correlated with the decline in glycemia (Figure 1J). In a third experimental series, gliflozins or vehicle were administered by oral gavage daily for 3 days to fed mice (Supplementary Figure S2A) and their blood was collected 16 h later. Glycemia was unaltered across the 3 days (Supplementary Figure S2B). Glucagonemia and insulinemia tended to decrease (Supplementary Figures S2C–D), but this was not exacerbated by the gliflozins (Supplementary Figures S2C–D: right panels). Moreover, none of the treatments affected the glucagon/insulin ratio (Supplementary Figure S2E).

3.2. SGLT2 inhibitors do not affect glucagon and insulin secretion

We evaluated whether gliflozins control islet hormone secretion directly.

3.2.1. Rats

Rat islets were incubated in the presence of 1 mM glucose to test the effects of empagliflozin, dapagliflozin (1–10 μM), and other agents used as controls on glucagon secretion. Although arginine, high K^+, and adrenaline significantly stimulated glucagon secretion, SGLT2i had no significant effect (Figure 2A).

3.2.2. Mice

The *in situ* perfused pancreas was used as the closest model to the *in vivo* situation. As commonly observed with this preparation [35–37], glucagon secretion decreased with time. As it has been claimed that the effect of gliflozins depends on the glucose concentration [19,20], we tested different protocols at various glucose (G) concentrations. Switching between 7 mM (G7) and 2 mM glucose (G2) (Figure 2B–E) or between G15 and G2 (Figure 2E) or the addition of 10 mM arginine (Figure 2F) strongly stimulated glucagon secretion. Dapagliflozin (1–10 μM), empagliflozin (1 μM), or sotagliflozin (1 μM) did not affect glucagon release and did not prevent the glucagonotropic effect of G2. Parallel measurements of insulin showed that G7, G15, and arginine stimulated insulin release (Figure 2B–F). In contrast, the gliflozins did not affect insulin secretion.

3.2.3. Humans

All these experiments were conducted on isolated islets. During static incubation experiments, glucagon secretion was lower at G11.1 than G1 and markedly stimulated by arginine and high K^+ (Figure 3A). However, neither empagliflozin nor dapagliflozin (10 μM) affected glucagon release. During dynamic perifusion experiments, switching from G10 to G0.5 strongly stimulated glucagon secretion and inhibited insulin release (Figure 3B–H). Dapagliflozin (1–10 μM), empagliflozin (1 μM), or sotagliflozin (1 μM) did not affect glucagon or insulin release and did not prevent the glucagonotropic effect of G0.5. The effect of the gliflozins was also tested at other glucose concentrations (Figure 4). Increasing the glucose concentration from 4 to 15 mM inhibited glucagon secretion and strongly stimulated insulin release (Figure 4A–C). In contrast, the gliflozins did not have significant effects on secretion of either hormone. All experiments described above were designed to test the effect of the gliflozins on glucagon secretion and were performed in the presence of amino acids that stimulate glucagon secretion. An additional series of experiments was performed in the absence of amino acids using a protocol that was designed to examine the potential effects of the gliflozins on insulin release. Switching from G7 to G15 induced a stimulation of insulin secretion that was reversed by the K_{ATP} channel opener, diazoxide, but further stimulated by high K^+ (Figure 4D–G). Elevating the glucose concentration from G7 to G15 or adding diazoxide had no effect on glucagon release; this was expected, as the glucagonostatic effect of glucose is known to be maximal at ~ G7 [38,39]. High K^+ stimulated glucagon release. However, none of the gliflozins (1 μM) affected glucagon or insulin release.

3.3. SGLT2 mRNA is not expressed in α- and β-cells

3.3.1. Gene expression measurements by qPCR

Three different methods, adapted to each species, were used to purify α- and β-cells by FACS (Supplementary Figure S1). The purity of both populations was excellent for all species, as insulin and glucagon mRNA were almost exclusively expressed in each of them (Figure 5A–B, D-E, and J-K). In rats, SGLT2 (*Slc5a2*) mRNA was absent in islet cells and in a series of other tissues except for kidney tissue, where it was highly expressed (Figure 5C, Supplementary

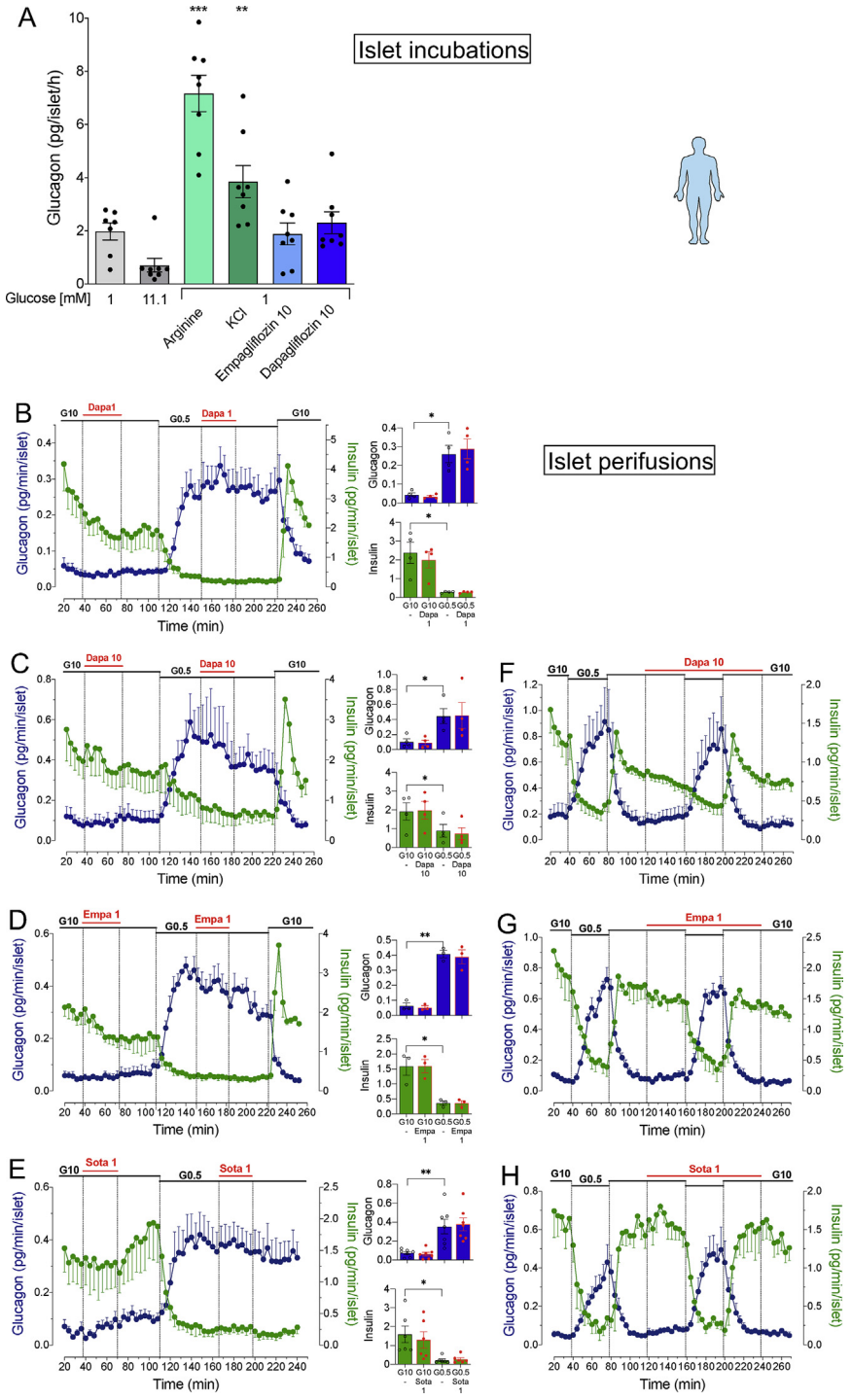

Figure 3: Gliflozins do not directly affect glucagon and insulin secretion from human islets. (*A*) Human islets (10 islets/100 μl medium) were incubated for 1 h in a medium containing 1 or 11.1 mM glucose and various test agents including arginine (10 mM), KCl (60 mM), empagliflozin (10 μM), or dapagliflozin (10 μM). Values are means ± SE for 7–8 batches of islets. $^{**}p < 0.01$ and $^{***}p < 0.001$ for comparisons between treatments and control (G1), ordinary one-way ANOVA followed by Fisher's LSD test for multiple comparisons. (*B–H*) Human islets (200–300 islets/chamber) were perifused with a solution containing a 6 mM mixture of three amino acids present at the following concentrations (in mM): 2 alanine, 2 glutamine, and 2 arginine. The glucose (G) concentration was changed between 10 and 0.5 mM, and dapagliflozin (Dapa, 1 or 10 μM), empagliflozin (Empa, 1 μM), and sotagliflozin (Sota, 1 μM) were applied when indicated. Traces are means ± SE of 3–7 experiments with different preparations. Graphs on the right of panels *B-E* are scatter-dot plots with bar graphs showing individual data and means ± SE of the average glucagon and insulin secretions calculated from the experiments shown on the left. Because glucagon secretion displayed spontaneous time-dependent changes, the mean secretion in the presence of the gliflozins was compared to the mean secretion integrated over the control period before and after the application of the gliflozins (G10 vs G10 + dapagliflozin or empagliflozin: mean of 20–36 and 76–108 min vs mean of 40–72 min; G0.5 vs G0.5 + dapagliflozin or empagliflozin: mean of 132–148 and 188–204 vs mean of 152–184 min; G10 vs G010 + sotagliflozin: mean of 20–36 and 92–108 min vs mean of 40–68 min; and G0.5 vs G0.5 + sotagliflozin: mean of 148–164 and 200–240 vs mean of 168–196 min). $^{*}p < 0.05$ and $^{**}p < 0.01$, paired t-test for comparison between different conditions.

Figure S3). In mice and humans, expression of glucose transporters other than SGLT2 was also measured for comparison (Figure 5F—I and L-O). In both species, SGLT2 mRNA was not detected in islet cells, whereas it was highly expressed in the kidney. It was also absent in a panel of other human tissues, except for a slight expression in the spleen (Supplementary Figure S3). SGLT1 (*Slc5a1*) mRNA was very poorly expressed in mouse and human β-cells, but more expressed in α-cells from humans than from mice, and strongly expressed in the duodenum (control tissue) of both species. GLUT1 (*Slc2a1*) and GLUT2 (*Slc2a2*) mRNA were more expressed in mouse and human β- than in α-cells. As expected, GLUT2 was much more expressed than GLUT1 in mouse β-cells, whereas the reverse was found in human β-cells.

3.3.2. Single-cell RNA-Seq analyses

We conducted two analyses of human single-cell RNA-Seq data from public domains (Figure 6 and Supplementary Figure S4). They confirmed our qPCR experiments and showed a moderate expression of SGLT1 in 25—50% of α-cells and no detectable expression of SGLT2 in the vast majority of islet cells. A modest expression of SGLT2 was observed in only 0.5—2.5% of α-cells, regardless of the gender or diabetic status (Figure 6C—D).

3.4. SGLT2 protein is not detectable in islet cells

3.4.1. Validation of the antibody

The anti-SGLT2 antibody was first validated on HEK293 cells expressing (HEK293-hSGLT2) or not expressing SGLT2. qPCR experiments confirmed that SGLT2 mRNA was not detected in HEK293 cells, whereas it was highly expressed in HEK293-hSGLT2 cells and the kidney (Figure 7A). Experiments based on the uptake of ^{14}C-α-methyl-D-glucopyranoside (αMG), a non-metabolizable glucose analogue specifically transported by SGLTs, showed that only HEK293-hsSGLT2 took up ^{14}C-αMG and that this uptake was fully prevented by empagliflozin (EC_{50}: 4.3 nM), the SGLT family inhibitor, phlorizin (EC_{50}: 41 nM), and Na^+ omission (Figure 7B).

In vivo, SGLT2 is highly glycosylated. We verified that the antibody raised against a 46 amino acid sequence (208—253) corresponding to an extracellular loop of human SGLT2 recognized equally well the glycosylated and unglycosylated protein. Membrane extracts of lysates from HEK293, HEK293-hSGLT2, and human kidney and islet cells were either pretreated or not pretreated with PNGaseF, a deglycosylation enzyme, before Western blotting. In keeping with the lack of SGLT2 mRNA expression in naïve HEK293 cells and islet cells, no band was detected by the anti-SGLT2 antibody (Figure 7C). In contrast, a clear band was observed in extracts from HEK293-hSGLT2 cells and the kidney. As expected, removal of glycosylation residues by PNGaseF induced a shift in the apparent molecular weight, but importantly, it did not affect the ability of the antibody to detect SGLT2. Immunodetections of SGLT2 were then conducted on fixed cells (Figure 7D). They revealed no signal in naïve HEK293 cells but a strong membrane labeling in HEK293-hSGLT2 cells, which indicated that the antibody specifically recognized SGLT2 in the fixed cells.

3.4.2. Immunodetections of SGLT2 in kidney and pancreatic sections

As expected, SGLT2 immunodetection showed a strong labeling restricted to proximal tubules in the kidneys of rats, mice, and humans (Figure 7E). Labeling was prevented by preincubation of the antibody with an SGLT2 peptide (not shown). Immunodetections were conducted on pancreatic sections including a total of 587 human, 305 rat, and 158 mouse islets (including 73,957 islet cells from 3 pancreas/species) (Figure 7F). β-, α-, and δ-cells were identified by antibodies against insulin, glucagon, and somatostatin, respectively. Immunodetection of SGLT2 on the same sections did not reveal any SGLT2 labeling in the three species. Pancreatic sections from patients with type 1 and type 2 diabetes were also negative for SGLT2 (not shown, n = 3/group).

3.5. SGLT inhibition does not affect glucagon gene expression

Rat and human islets were treated for 24 h with 10 μM empagliflozin or dapagliflozin in the presence of two different glucose concentrations adapted to the species (G11 and G25 for rats and G5 and G15 for humans; Figure 8). Neither SGLT2i affected glucagon gene expression under any of the tested conditions.

4. DISCUSSION

We showed that SGLT2 inhibition in mice increases the plasma glucagon/insulin ratio in the fasted state, an effect correlated with a decline in glycemia. However, functional experiments on pancreatic tissue from rats, mice, and humans did not reveal any direct effect of SGLT1/2 inhibitors on glucagon and insulin secretion. Gene expression analysis and immunodetection showed no detectable SGLT2 expression in the islet cells of all three species. However, we found modest SGLT1 gene expression in human α-cells only. SGLT2 inhibition also had no effect on glucagon gene expression. The data strongly suggest that the increase in the plasma glucagon/insulin ratio induced by SGLT1/2i *in vivo* does not result from a direct action of the gliflozins on islet cells, but is likely secondary to their insulin-independent glucose-lowering effects.

4.1. SGLT2 inhibition increases the plasma glucagon/insulin ratio in the fasted state

The gliflozins (dapagliflozin, empagliflozin, and sotagliflozin) tested in this study were very effective, as they strongly increased glucosuria. Several reports have shown that gliflozins increase glucagonemia or decrease insulinemia [3,8,14,19,23]. We replicated these observations as administering dapagliflozin (1—10 mg/kg BW) to fasted mice produced, 1 h later, a dose-dependent increase in glucagonemia and the plasma glucagon/insulin ratio. These changes correlated with a decline in glycemia. One mg/kg BW of dapagliflozin induces a plasma C_{max} of ∼500 nM in mice [40], which is close to the plasma C_{max} of ∼400 nM in humans who are receiving a therapeutic dose of 10 mg [41]. However, contrary to reports showing increased glucagonemia 6 h or 18 h after dapagliflozin administration [14,19], we did not find any alteration in the glucagon/insulin ratio 16 h after three daily doses (1 mg/kg BW) of dapagliflozin, empagliflozin, or sotagliflozin. This lack of effect might be explained by the relatively rapid clearance of the gliflozins [42,43].

4.2. SGLT2 is not expressed in islet cells of rats, mice, and humans

We measured *SGLT2* gene expression in FACS-purified populations of α- and β-cells from rats, mice, and humans. In the three species, *SGLT2* expression was below detection levels in both α- and β-cells, whereas it was highly expressed in the kidney. In mice and humans, we also measured the gene expression of *SGLT1*. Interestingly, *SGLT1* was barely detectable in mouse α- and β-cells, whereas it was well expressed in human α-cells. Complementary analysis of two single-cell RNA-Seq datasets confirmed our qPCR analysis, in agreement with a recent study [17]. In particular, no expression of *SGLT2* was

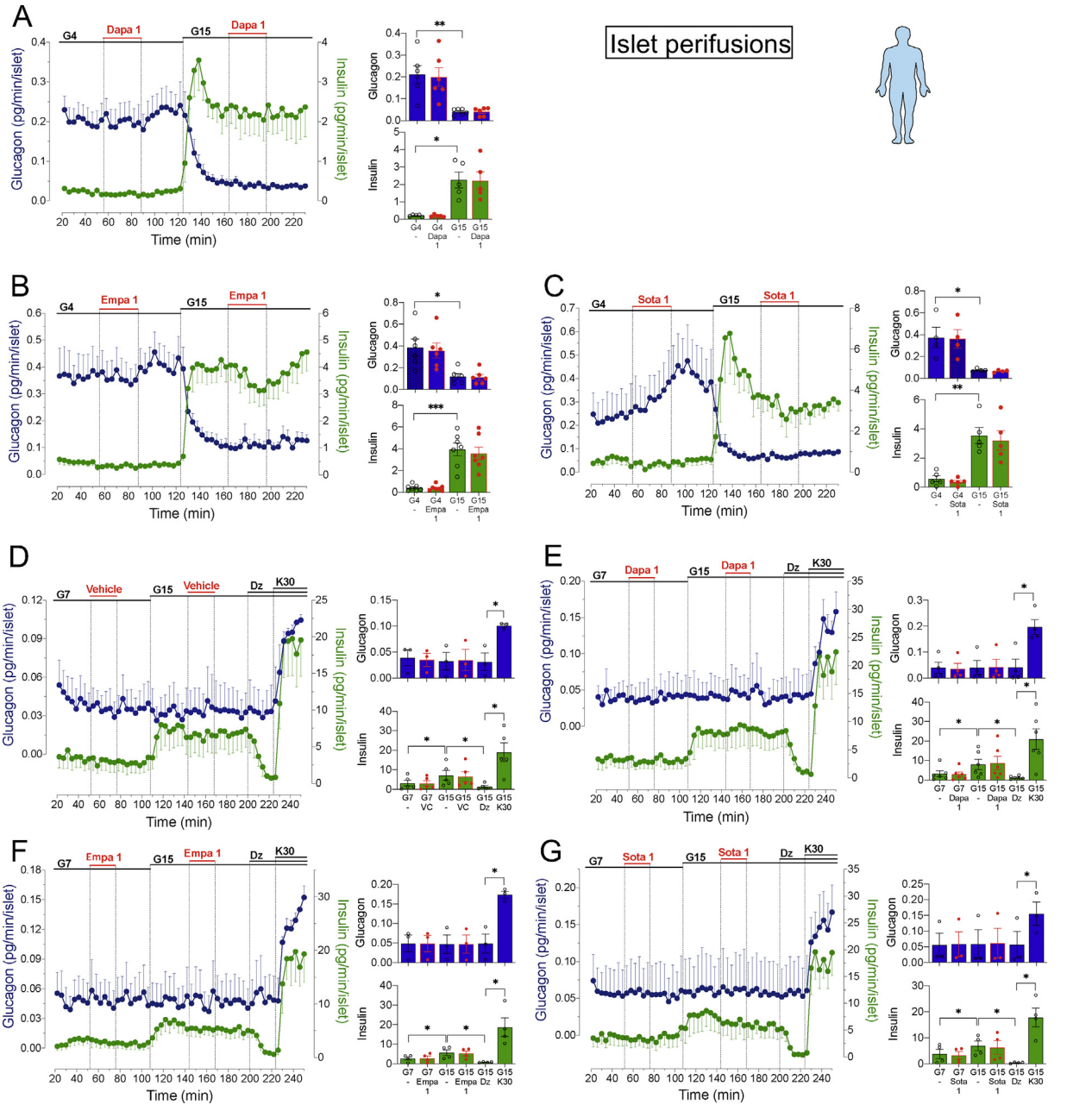

Figure 4: Gliflozins do not directly affect glucagon and insulin secretion from human islets. (*A–C*) Human islets (~200–300 islets/chamber) were perifused with a solution containing a 6 mM mixture of three amino acids present at the following concentrations (in mM): 2 alanine, 2 glutamine, and 2 arginine. The glucose (G) concentration was changed between 4 and 15 mM, and dapagliflozin (Dapa, 1 μM), empagliflozin (Empa, 1 μM), and sotagliflozin (Sota, 1 μM) were applied when indicated. Traces are means ± SE of 3–6 experiments with different preparations. Graphs on the right of panels *A–C* are scatter-dot plots with bar graphs showing individual data and means ± SE of the average glucagon and insulin secretions calculated from the experiments shown on the left. The mean secretion in the presence of the gliflozins was compared to the mean secretion integrated over the control period before and after the application of the gliflozins (G4 vs G4 + gliflozin: mean of 20–52 and 88–120 min vs mean of 56–84 min; G15 vs G15 + gliflozin: mean of 148–160 and 196–228 vs mean of 164–192 min). In panel *C*, the trend in glucagon secretion increase induced by sotagliflozin in G4 was not significant when comparing the mean of 20–52 min vs the mean of 56–84 min ($p = 0.0651$). (*D-G*) Human islets (~300–400 islets/chamber) were perifused with an amino acid-free solution. The glucose (G) concentration was changed between 7 and 15 mM, and vehicle (DMSO 0.002%), dapagliflozin (1 μM), empagliflozin (1 μM), sotagliflozin (1 μM), diazoxide (Dz, 250 μM), and KCl (30 mM, K30) were added when indicated. Traces are means ± SE of 3–6 experiments with different preparations. Graphs on the right of panels *D-G* are scatter-dot plots with bar graphs showing individual data and means ± SE of the average glucagon and insulin secretions calculated from the experiments shown on the left. The mean secretion in the presence of vehicle or gliflozins was compared to the mean secretion integrated over the control period before and after the application of the test agent (G7 vs G7 + vehicle or gliflozin: mean of 20–48 and 76–104 min vs mean of 52–72 min; G15 vs G15 + vehicle or gliflozin: mean of 120–140 and 168–196 vs mean of 144–164 min; and G15 Dz vs G15 K30: mean of 212–220 min vs mean of 240–248 min). *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$, paired t-test for comparison between different conditions.

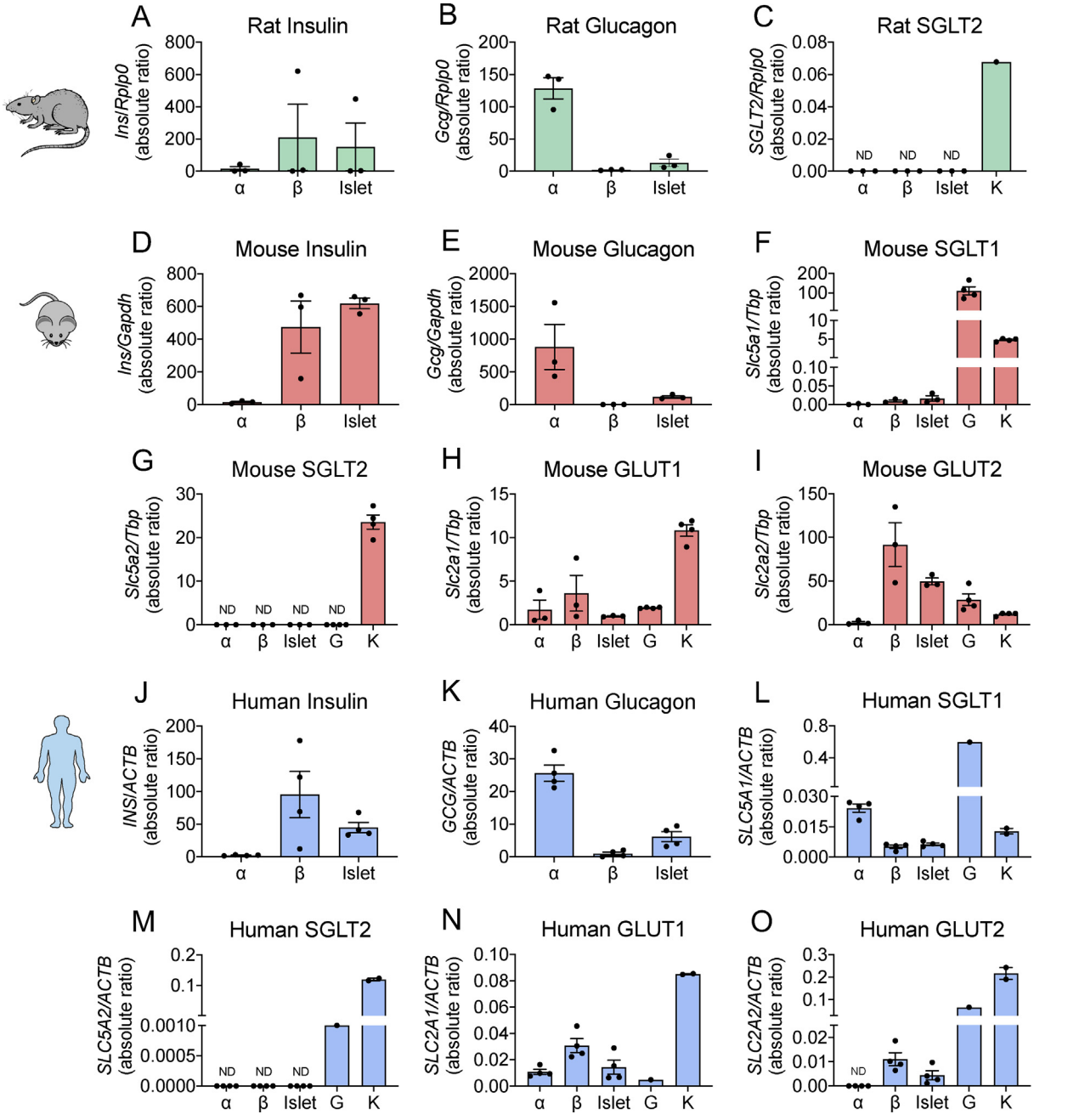

Figure 5: The SGLT2 gene is not expressed in rat, mouse, or human pancreatic islet cells. (*A—B*) Insulin (*Ins*) and glucagon (*Gcg*) gene expression in rat FAC-sorted α- and β-cells and pancreatic islets normalized to *Rplp0*. (*C*) SGLT2 gene expression in rat α-cells, β-cells, pancreatic islets, and kidney (K) normalized to *Rplp0* (n = 3 for islets, α-cells, and β-cells; n = 1 for K). (*D—E*) Insulin (*Ins*) and glucagon (*Gcg*) gene expression in mouse FAC-sorted α- and β-cells and pancreatic islets normalized to *Gapdh*. (*F—I*) SGLT1 (*Slc5a1*), SGLT2 (*Slc5a2*), GLUT1 (*Slc2a1*), and GLUT2 (*Slc2a2*) gene expression in mouse α-cells, β-cells, pancreatic islets, gut (G), and kidney (K) normalized to *Tbp* (n = 3 for islets, α-cells, and β-cells; n = 4 for G and K). (*J-K*) Insulin (*INS*) and glucagon (*GCG*) gene expression in human FAC-sorted α-and β-cells and pancreatic islets normalized to actin (*ACTB*). (*L-O*) SGLT1 (*SLC5A1*), SGLT2 (*SLC5A2*), GLUT1 (*SLC2A1*), and GLUT2 (*SLC2A2*) gene expression in human α-cells, β-cells, pancreatic islets, gut (G), and kidney (K) normalized to actin (*ACTB*) (n = 4 for islets, α-cells, and β-cells; n = 1 for G; n = 2 for K). All the graphs show individual data and means ± SE. Genes with Ct values above 38 for rats and 40 for mice and humans were considered non-detected (ND).

detected in the vast majority of human α-, β-, or δ-cells. We searched for the presence of SGLT2 protein in islet cells by conducting immunodetections with an antibody raised against a 46 amino acid sequence of SGLT2 that shares only 45% homology to SGLT1. This antibody produced a strong labeling in proximal tubules in the kidney, but did not stain any cells in pancreatic sections including 587 human and 463 rodent islets. In summary, these data do not support the presence of SGLT2 mRNA or protein at levels of functional relevance in α-, β-, or δ-cells as reported by others [19,21,25].

4.3. Gliflozins do not directly affect glucagon and insulin secretion or glucagon gene expression

To evaluate if gliflozins directly control hormone secretion from islets, we tested their effects on glucagon and insulin secretion from isolated

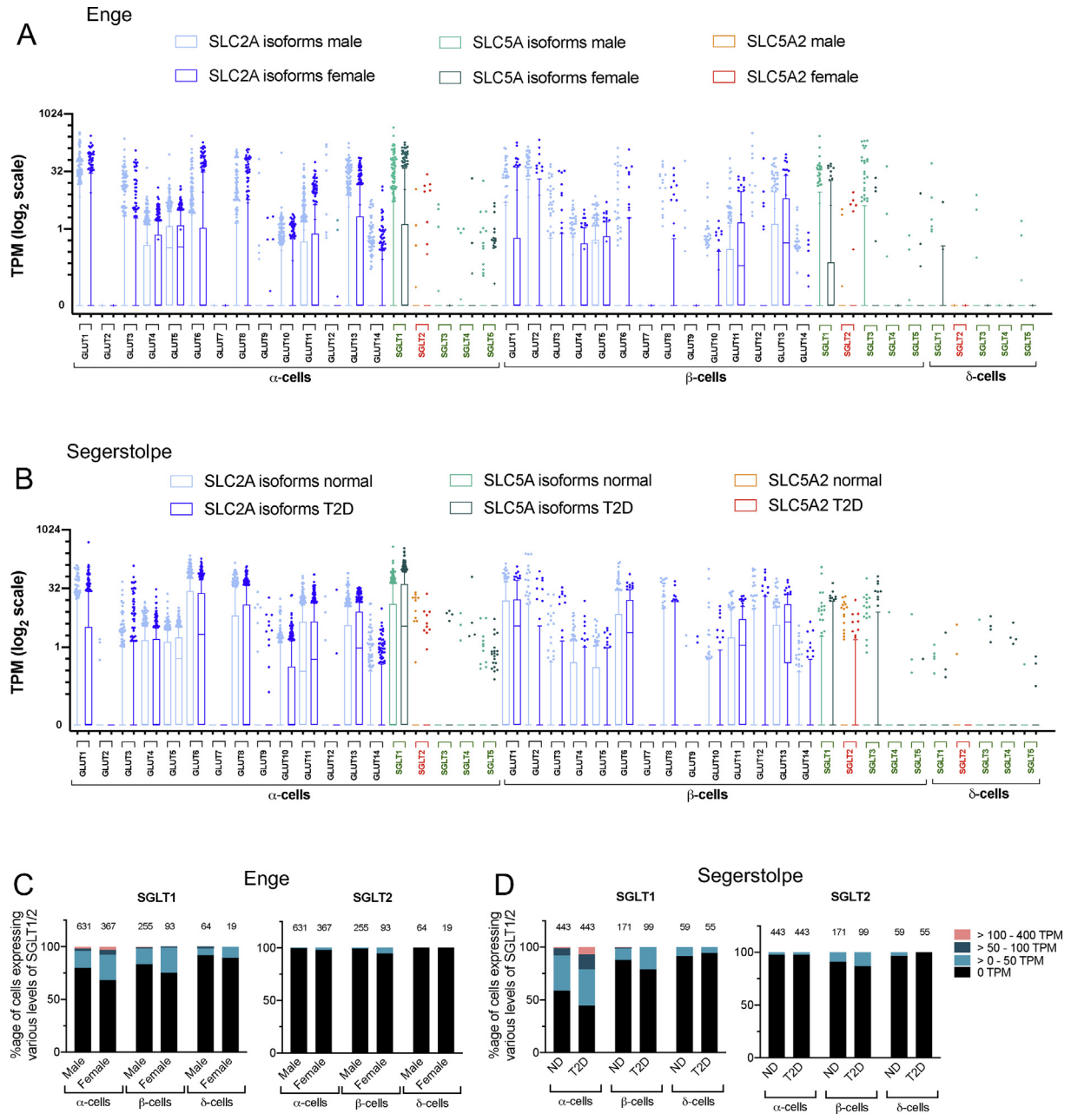

Figure 6: Human islet cells express various glucose transporters but almost no SGLT2. (*A*–*B*) Single-cell RNA-Seq expression analysis of various glucose transporters in pancreatic α-, β-, and δ-cells from male and female donors from Enge et al. (*A* [31]) and diabetic and healthy donors from Segerstolpe et al. (*B* [32]) in transcripts per million (TPM). A pseudocount of 0.1 was added to the actual TPM values before plotting. Results are shown as boxes and whiskers (10–90% percentile). (*C*–*D*) Percentage of cells expressing SGLT1 and SGLT2 at different expression levels in Enge's (*A*) and Segerstolpe's (*B*) data.

rat or human islets and the *in situ* perfused mouse pancreas. We used concentrations well above the IC_{50} of gliflozins for SGLT2 and the drugs' therapeutic C_{max}. The IC_{50} of empagliflozin, dapagliflozin, and sotagliflozin for hSGLT2/mSGLT2 were 3.1/1.9 nM [33,44,45], 1.2/2.3 nM [33], and 1.8/0.6 nM [46,47], respectively. The plasma C_{max} in humans is 500–700 nM for 25 mg empagliflozin/day [42], ~400 nM for 10 mg dapagliflozin/day [41], and ~500 nM for 300 mg sotagliflozin/day [41]. As all gliflozins strongly bind to plasma proteins (86% for empagliflozin [48], 91% for dapagliflozin [41,49], and 98% for sotagliflozin [50]), the free plasma C_{max} of the gliflozins does not exceed 100 nM (700 nM x 0.14 = 98 nM for empagliflozin, 400 nM x 0.09 = 36 nM for dapagliflozin, and 500 nM x 0.02 = 10 nM for sotagliflozin), which are, for all, at least 5 times more than their respective IC_{50} for SGLT2. The minimal gliflozin concentration that we used was 1 μM. As we used a medium that contained at least 50 times lower protein concentration than plasma, it was expected that the free gliflozin concentrations in our test conditions were above 500 nM, that is, at least 5 times higher than their free plasma C_{max} when used at therapeutic doses and 150 times higher than their IC_{50} for SGLT2. However, even at these supratherapeutic concentrations, we did not see any significant effect of gliflozins on glucagon and insulin secretion from islets or perfused pancreas of the three species tested.

We did not test the effect of gliflozins on somatostatin secretion because our results did not support the possibility that the gliflozins

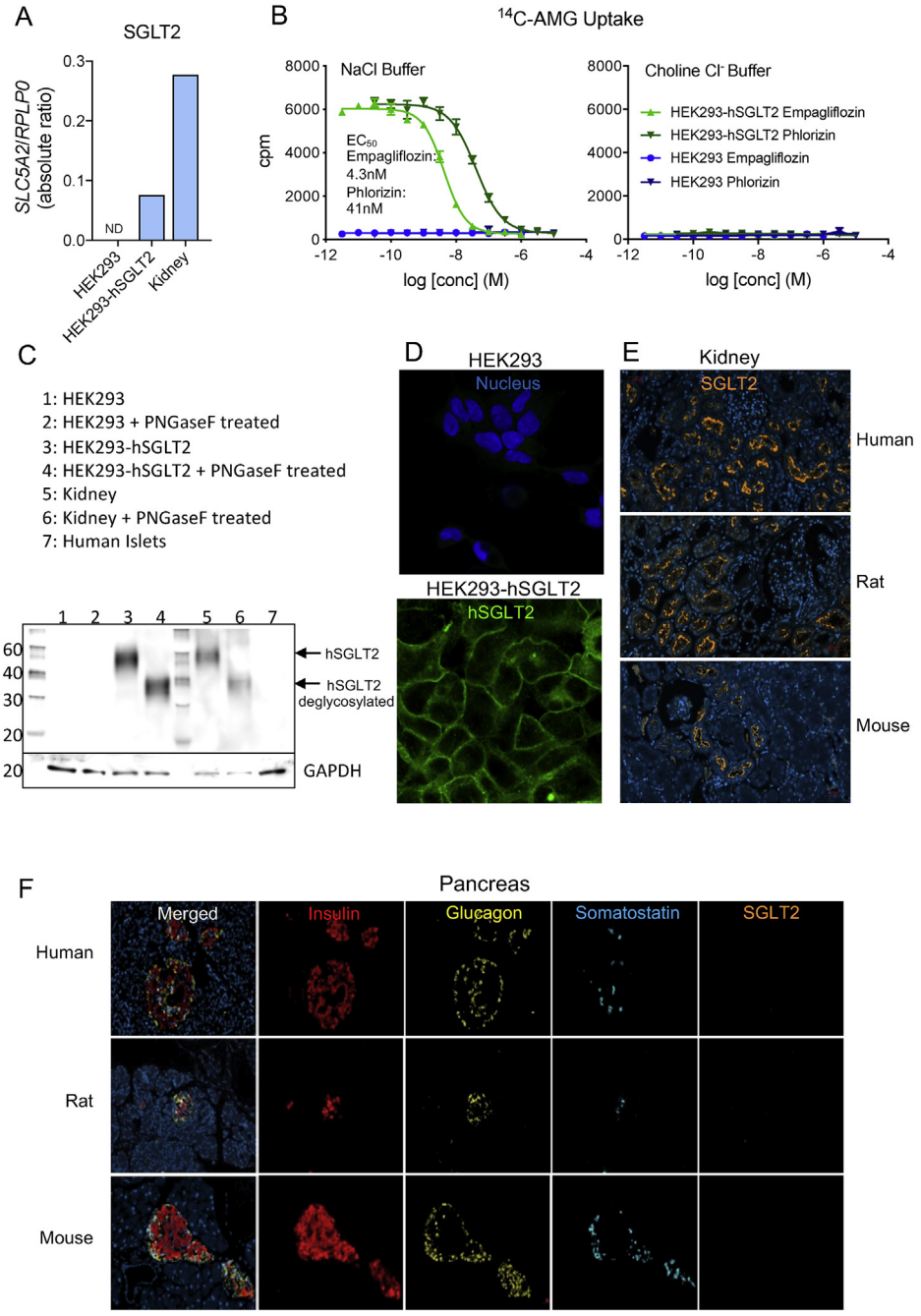

Figure 7: SGLT2 protein is highly expressed in the kidney but undetectable in islet cells. (*A*) qPCR of SGLT2 gene expression in HEK293 cells expressing (HEK293-hSGLT2) or not SGLT2, and in human kidney normalized to RPLP0 (n = 2 for HEK cells and n = 1 for kidney). It shows that naïve HEK293 cells do not express significant levels of SGLT2 compared to HEK293-hSGLT2 and the kidney. (*B*) Uptake of ^{14}C-α-methyl-D-glucopyranoside (^{14}C-AMG) in HEK293 cells expressing (HEK293-hSGLT2) or not SGLT2. The left panel shows that only HEK293-hSGLT2 uptakes ^{14}C-αMG and that this uptake is fully prevented by empagliflozin and phlorizin. The right panel shows that, as expected for SGLT, this uptake is Na^+-dependent because it was prevented by replacement of Na^+ by choline. Data are means ± SE of 4 repetitions. (*C*) Validation of the anti-SGLT2 antibody by Western blotting. Membranes of HEK293 cells expressing (HEK293-hSGLT2, bands 3 and 4) or not (bands 1 and 2) SGLT2, human kidney (bands 5 and 6), and islets (band 7) were pretreated or not with PNGaseF (a deglycosylation enzyme) for 1 h at 37 °C before being processed for Western blotting using the anti-SGLT2 antibody. As expected, PNGaseF removed all glycosylation residues on SGLT2 and induced a shift in the molecular weight. Loading of the gel was controlled by Western blotting for GAPDH. Although the loading of islet extract exceeded that of control tissues, no SGLT2 band was detected in the islets. The blotting is representative of 7 repetitions for HEK293/HEK293-hSGLT2 cells +/− PNGaseF, 5 repetitions for kidney +/− PNGaseF, and 1 blot for islets. (*D*) Immunodetection of SGLT2 on HEK293 cells expressing (HEK293-hSGLT2 cells) or not SGLT2. SGLT2 immunolabeling was restricted to the plasma membrane of HEK293-SGLT2 cells, while no signal was observed in normal HEK293 cells that were visualized by labeling their nuclei in blue via DAPI. (*E*) Immunodetection of SGLT2 in human, rat, and mouse kidney sections shows the expected labeling in proximal tubules. (*F*) Human, rat, and mouse pancreatic sections were processed for multiplex immunolabeling of insulin, glucagon, somatostatin, and SGLT2 on the same sections. No SGLT2 signal was detected in pancreatic sections containing β-cells (insulin), α-cells (glucagon), and δ-cells (somatostatin). Nuclei visualized on merged images were labeled with DAPI. Images are representative of 587 human, 305 rat, and 158 mouse islets.

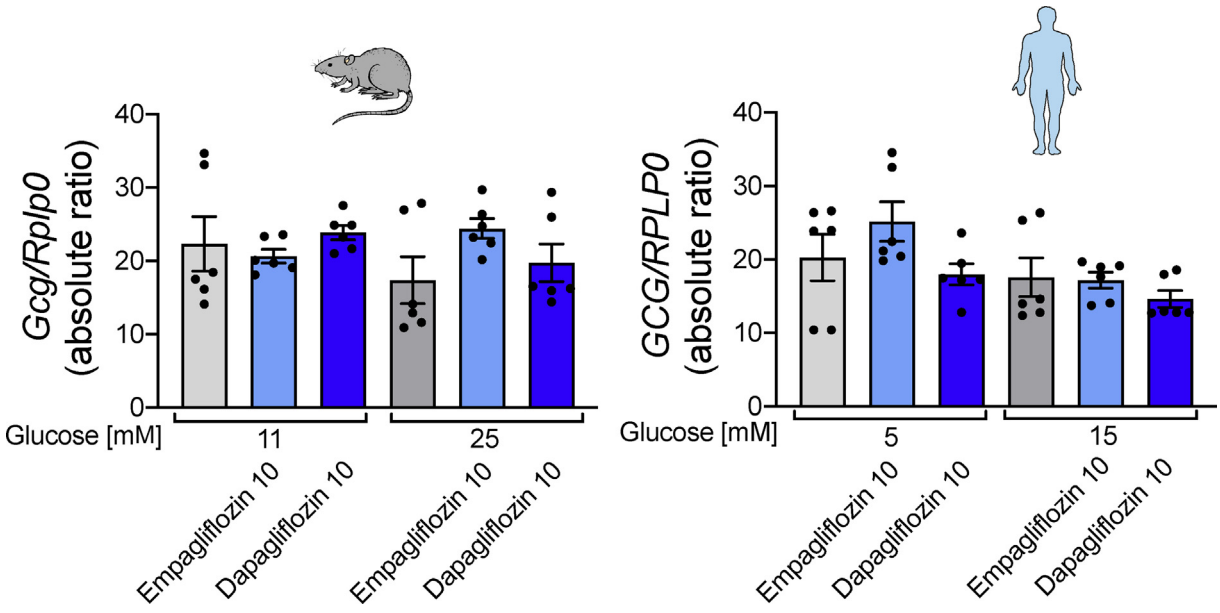

Figure 8: Empagliflozin and dapagliflozin do not modulate glucagon gene expression in human or rat islets. Glucagon mRNA expression (normalized to *Rplp0*/*RPLP0*) in rat and human pancreatic islets treated with empagliflozin (10 μM) or dapagliflozin (10 μM) in the presence of 11 or 25 mM (rat islets) or 5 or 15 mM of glucose (human islets) as indicated (n = 6). All the graphs show individual data and means ± SE. Groups were not significantly different, as tested by ordinary one-way ANOVA followed by Tukey's multiple comparison.

might indirectly control islet hormone secretion, as suggested by others [25]. However, others failed to observe an effect of dapagliflozin on somatostatin release [17].

It has been suggested that canagliflozin, a gliflozin with a weak inhibitory effect on SGLT1, stimulates glucagon release by targeting SGLT1 in α-cells [23]. Although our gene expression analyses support the expression of SGLT1 in human α-cells, we did not observe any effect of 1 μM sotagliflozin, a dual SGLT1/2 inhibitor, on glucagon secretion of human islets. This concentration far exceeds the IC_{50} of the drug for hSGLT1 (36 nM) [46]. This lack of effect was surprising because it was expected that the characteristics of SGLT1 (cotransport of glucose with 2 Na^+ and K_m of 2 mM [51]) help to concentrate glucose inside the cell and that SGLT1 inhibition would affect glucose fluxes across the plasma membrane. However, given the relatively low expression of *SGLT1* in α-cells (vs the gut), it is possible that other glucose transporters, such as GLUT1 or GLUT11, which have a high affinity for glucose, are expressed at the plasma membrane and are present in human α-cells [18,31,32], keeping glucose flux unaltered in our experimental conditions when SGLT1 was inhibited. It is also unknown whether the *SGLT1* transcript in α-cells generates a functional protein. In that context, it would be very surprising to see an Na^+-dependent glucose transport in α-cells that are, at first sight, not polarized, whereas most of the SGLTs are expressed in polarized epithelia of specific organs, such as the gut or the kidney, to actively take up glucose against its concentration gradient. Moreover, it is questionable why the α-cell, which needs to adapt its secretion rate to the external glucose concentration, would use a transporter that concentrates glucose to intracellular levels that do not reflect the extracellular levels.

It has also been suggested that SGLT2i stimulate glucagon gene expression and that this could contribute to hyperglucagonemia [19]. However, we did not detect any effect of 10 μM empagliflozin or dapagliflozin on glucagon gene expression in rat and human islets.

Discrepancies between previously published studies have sometimes been attributed to differences between the types and concentrations of the gliflozin used, species (mouse, rat, or human), cell types (primary cells or cell lines), experimental models (isolated islets, perfused pancreas, and *in vivo* models), conditions (glucose concentration or other agents), techniques/methods (antibodies or type of gene expression analysis), and, more recently, inter-individual heterogeneity [21]. However, one strength of our study is that the experiments were conducted on three different species using multiple approaches and all of our findings converged to the same conclusion.

5. CONCLUSION

Our study confirms observations that gliflozins increase the plasma glucagon/insulin ratio, at least in the fasted state, and provides strong evidence that this effect does not result from a direct action on islets. We therefore propose that the SGLT2i-induced increase in glucagonemia is a physiological metabolic adaptation to counteract the glucose-lowering effect of the drugs. The increase in energy intake upon SGLT2 inhibition also points in a similar direction, whereby a negative energy balance has to be counteracted by increased gluconeogenesis and energy intake to re-establish homeostasis.

Of note, while the SGLT2i-induced increase in the glucagon/insulin ratio likely contributes to enhanced EGP, it is probably not the only mechanism. This is supported by a recent study showing increased dapagliflozin-induced EGP in humans in conditions in which glucagon and insulin secretions were clamped at basal levels by somatostatin [52]. There is also an ingrained belief that glucagon exerts strong ketogenic activities, particularly during fasting and insulinopenic conditions. Hence, a strong increase in the plasma glucagon/insulin ratio has often been considered a major cause of diabetic ketoacidosis. However, the implication of glucagon in ketosis needs to be carefully reevaluated, since a recent study reported that neither fasting nor SGLT2i-induced ketosis are altered by interruption of glucagon signaling in mice unless there is a complete loss of insulin signaling [14]. Another study suggested that the SGLT2i-induced increased

incidence of ketoacidosis is independent from hyperglucagonemia but results, in an insulinopenia setting, from increased plasma catecholamine and corticosterone concentrations secondary to volume depletion [11]. Our data, together with these considerations, should temper the concerns that glucagon is a major aggravating cause of EGP and ketoacidosis when using SGLT2i.

AUTHORS' CONTRIBUTIONS

P.G. and R.A. conceived the study and designed the experiments. P.G. wrote the manuscript. H.C., E.G., F.K., M.B., N.A., B.K.L., D.B., B.Si., L.R., B.St., and H.K. conducted and designed the experiments and analyzed the results. A.W., C.B., N.R., L.P., F.G., F.R., and P.L.H. provided material. H.C., E.G., M.B., B.St., H.K., E.M., F.G., and F.R. contributed to the discussion. All coauthors approved the manuscript. P.G. and R.A. are guarantors of this work and as such have full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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PRIOR PRESENTATION

Parts of this study were presented at the 55th EASD annual meeting, Barcelona, Spain, September 16–20, 2019.

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CONFLICT OF INTEREST

R.A., E.M. B.St., H.K., M.P., and M.M. are employees of Boehringer Ingelheim Pharma GmbH & Co. KG. Biberach, Germany. The remaining authors declare that they have no conflicts of interest in connection with this manuscript.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.molmet.2020.101071.

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