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Abstract

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Reference

CHAE, Heeyoung, et al. 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. Molecular Metabolism, 2020, p. 101071

DOI: 10.1016/j.molmet.2020.101071
PMID: 32896668
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PII: S2212-8778(20)30145-9
DOI: https://doi.org/10.1016/j.molmet.2020.101071
Reference: MOLMET 101071
To appear in: Molecular Metabolism

Received Date: 30 July 2020
Revised Date: 13 August 2020
Accepted Date: 25 August 2020


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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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Running title: No direct effect of SGLT2i on α- and β-cells

Keywords: Gliflozins; SGLT2 inhibitor; glucagon; insulin; diabetes

Abstract word count: 207

Word count: 4123/8696

Number of figures: 8

Online Supplementary material: 4 Supplementary Figures + 2 Supplementary Tables

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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. However, they 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. Here, we aimed to verify whether or not SGLT2i affect glucagon and insulin secretion by direct action on islet cells in three species using multiple approaches.

Methods and Results: SGLT2 inhibition in mice increased the plasma glucagon/insulin ratio in the fasted state, an effect correlated with a drop in glycemia. Gene expression analyses and immunodetections showed no SGLT2 mRNA or protein expression in rodent and human islet cells, but a moderate SGLT1 mRNA expression in human α-cells. However, functional experiments on rat, mouse, and human (29 donors) islets and with the in situ perfused mouse pancreas did not reveal any direct effect of selective SGLT2i (dapagliflozin, empagliflozin) and a SGLT1/2i (sotagliflozin) on glucagon and insulin secretion. SGLT2i did not affect glucagon gene expression in rat and human islets.

Conclusions: These 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.

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

SGLT2 inhibitors (SGLT2i), named 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 increase in lipolysis and 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 the 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 drop 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 which express SGLT2 [19-22]. Others suggest 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 by a direct action on islet cells. To identify potential interspecies differences that might explain the controversies, this study was performed 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 data sets. 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 approvals

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

2.2 Models and tissue preparations

*Rodent strains and islet preparations:* Wistar-Han rats and C57BL/6N mice (6–12 months) were used for all experiments, except for gene expression, which was done 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.

*Human islets:* The origin and characteristics of the human islet preparations are listed in [Supplementary Table S1](#). After shipment, 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

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

**cDNA preparation:** RNA was extracted using Dynabead-oligodT or TriPure and reverse transcribed into cDNA.

**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 the inferred cell type annotation provided with the respective entries to visualize gene expression per cell type.

2.5 $^{14}$C-αMG uptake experiments and Western blot analysis for SGLT2

$^{14}$C-αMG uptake experiments were performed as previously described [33]. The SGLT2 antibody was characterized by Western blot analysis using total membrane extracts of lysates from human islets and kidneys, and HEK293 cells expressing or not SGLT2. Western blot analysis and treatment with PNGaseF 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 Opal Polymer Anti-Mouse and Rabbit HRP Kit (Perkin Elmer).
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).

### 2.8 Secretion experiments

**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 CaCl₂, 0.81
MgSO₄, 0.34 NaH₂PO₄, 0.44 KH₂HPO₄ + 1 mg/mL BSA, pH 7.4. Islets were kept for 30 min
in 25 (rat) or 11.1 mM (human) glucose, before being transferred in 1 mM glucose containing
the respective treatments. One hour later, glucagon was determined using the Fluorescent EIA
Kit (Phoenix Pharmaceuticals).

**Dynamic secretion experiments:** Experiments on perifused mouse and human islets and with
the perfused mouse pancreas were performed as previously described [35]. The medium
contained (in mM): 124 NaCl, 4.8 KCl, 2.5 CaCl₂, 1.2 MgCl₂, 20 NaHCO₃ + 1 mg/mL BSA,
PH 7.4. Except where otherwise indicated, it was supplemented with a 6 mM mixture (for
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
radioimmunoassay.

### 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 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 to mice kept for 16 h in metabolic cages and their urine collected (Figure 1A). Glycemia tended to decrease during this 16-h period but the gliflozins did not exacerbate this drop (Figure 1B–C). As expected, all 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. Therefore, mice were fasted for 16 h (to be in a condition where glucagonemia is increased) before receiving a single dose of either vehicle or the gliflozin (Figure 1E), and 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 drop 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 blood was collected 16 h later. Glycemia was unaltered across the 3 days (Supplementary Figure S2B). Glucagonemia and insulinemia tended to decrease (Supplementary Figure S2C–D), but this was not exacerbated by the gliflozins (Supplementary Figure 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 if the gliflozins control islet hormone secretion directly.

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 control on glucagon secretion. Although arginine, high K\(^+\), and adrenaline significantly stimulated glucagon secretion, SGLT2i were without significant effect (Figure 2A).

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 is dependent on 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 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). By contrast, the gliflozins did not affect insulin secretion.

Humans: All these experiments were performed on isolated islets. During static incubation experiments, glucagon secretion was lower at G11.1 than G1 and was 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). By 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 were performed in the absence of amino acids using a protocol that was designed to see 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<sub>ATP</sub> channel opener, diazoxide, but further stimulated by high K<sup>+</sup> (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 already maximal at ~G7 [38,39]. High K<sup>+</sup> 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

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, 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 S3). In mice and humans, expression of glucose transporters other than SGLT2 was also measured for comparison (Figure 5F–I, 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 was more expressed in α-cells from humans than from mice, and it was 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 α-cells. As expected, GLUT2 was much more expressed than GLUT1 in mouse β-cells, whereas the reverse was found in human β-cells.

**Single-cell RNA-Seq analyses:** We performed 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 the diabetic status (Figure 6C–D).

### 3.4 SGLT2 protein is not detectable in islet cells

**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 kidney (Figure 7A). Experiments based on the uptake of $^{14}$C-$\alpha$-methyl-D-glucopyranoside ($\alpha$MG), a non-metabolizable glucose analogue specifically transported by SGLTs, showed that only HEK293-hSGLT2 take up $^{14}$C-$\alpha$MG, 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 recognizes 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 performing Western blots. 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). By 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 of the apparent molecular
weight, but importantly, it did not affect the ability of the antibody to detect SGLT2.
Immunodetections of SGLT2 were then performed on fixed cells (Figure 7D). They revealed
no signal in naïve HEK293 but a strong membrane labeling in HEK293-hSGLT2, which
indicates that the antibody specifically recognizes SGLT2 in fixed cells.

**Immunodetections of SGLT2 in kidney and pancreatic sections:** As expected, SGLT2
immunodetection showed a strong labeling restricted to proximal tubules in the kidney of rats,
mice, and humans (Figure 7E). The labeling was prevented by preincubation of the antibody
with a SGLT2 peptide (not shown). Immunodetections were performed 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 diabetes 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 in mice that SGLT2 inhibition increases the plasma glucagon/insulin ratio in the
fasted state, an effect correlated with a drop 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 immunodetections showed no detectable SGLT2 expression in islet cells of all three species. However, we found a modest SGLT1 gene expression in human α-cells only. SGLT2 inhibition was also without effect on glucagon gene expression. These data strongly suggest that the increase in 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 administration of 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 – changes that were correlated with a drop in glycemia. 1 mg/kg BW of dapagliflozin induces a plasma C\text{max} of ~500 nM in mice [40], which is close to the plasma C\text{max} of ~400 nM seen 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 of the glucagon/insulin ratio 16 h after 3 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 level in both α- and β-cells, whereas it was highly expressed in the kidney. For mice and humans, we
also measured 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 detected in the vast majority of human α-, β- or δ-cells. Finally, we searched for the presence of the SGLT2 protein in islets cells by performing immunodetections with an antibody raised against a 46 amino acids 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 cell in pancreatic sections including 587 human and 463 rodent islets. In summary, these data do not support a 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 the gliflozins directly control hormone secretion from islets, we tested their effects on glucagon and insulin secretion from isolated rat or human islets and from the in situ perfused mouse pancreas. We used concentrations well above the IC$_{50}$ of the gliflozins for SGLT2 and the therapeutic C$_{max}$ of the drugs. The IC$_{50}$ of empagliflozin, dapagliflozin and sotagliflozin for hSGLT2/mSGLT2 are, respectively, 3.1/1.9 nM [33,44,45], 1.2/2.3 nM [33] and 1.8/0.6 nM [46,47]. 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 of them, at least 5x more than their respective IC$_{50}$ for SGLT2.
The minimal concentration of the gliflozins that we used was 1 µM. As we used a medium which contained at least 50 times lower protein concentration than plasma, it is expected that the free gliflozin concentrations in our test conditions were above 500 nM, i.e. at least 5x times higher than their free plasma C\text{max} when used at therapeutic doses and 150x times higher that their IC\text{50} for SGLT2. However, even with 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 the gliflozins on somatostatin secretion because our results do not support the possibility that the gliflozins might indirectly control islet hormone secretion as suggested by others [25]. However, others failed to see 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 see any effect of 1 µM sotagliflozin, a dual SGLT1/2 inhibitor on glucagon secretion of human islets. This concentration far exceeds the IC\text{50} of the drug for hSGLT1 (36 nM) [46]. This lack of effect is surprising because it is expected that the characteristics of SGLT1 (cotransport of glucose with 2Na\textsuperscript{+}, and K\textsubscript{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 (versus 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], keep glucose flux unaltered in our experimental conditions when SGLT1 is 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 a Na\textsuperscript{+}-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 ones.

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 rats and human islets.

Discrepancies between previously published studies have sometimes been attributed to differences between the types and the concentrations of the gliflozin used, species (mouse, rat, human), cell types (primary cells or cell lines), experimental models (isolated islets, perfused pancreas, in vivo models), conditions (glucose concentration or other agents), techniques/methods (antibodies, type of analysis of gene expression), and, more recently, to inter-individual heterogeneity [21]. However, one strength of our study is that the experiments were performed on three different species, using multiple approaches, and that all our findings converge 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 evidences that this effect does not result from a direct action on the 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. Similarly, the increase in energy intake upon SGLT2 inhibition is pointing in a similar direction, whereby a negative energy balance has to be counteracted by increased gluconeogenesis and energy intake to re-establish homeostasis.

It is worth mentioning that 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 where 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 in 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, here also, 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 has suggested that the SGLT2i-induced increased incidence of ketoacidosis is independent from hyperglucagonemia but results, in the setting of insulinopenia, from increased plasma catecholamine and corticosterone concentrations secondary to volume depletion [11]. Our data, together with these considerations, should tune down the fear that glucagon is a major aggravating cause of EGP and ketoacidosis when using SGLT2i.

ACKNOWLEDGMENTS

Some human pancreatic islets were provided through the JDRF award 31-2008-416 (ECIT Islet for Basic Research program) or by the NIDDK-funded integrated Islet Distribution (IIDP) at City of Hope, NIH Grant #2UC4DK098085. We thank JC Jonas (Université Catholique de Louvain, Brussels, Belgium) and R Chimienti (San Raffaele Scientific Institute, Milan, Italy) for advices on gene expression, and F Knockaert for help for radioimmunoassays. We thank O Devuyst (University of Zurich, Zurich, Switzerland) and I Leclercq (Université Catholique de Louvain, Brussels, Belgium) for the gift of cDNA from human kidney and duodenum, respectively. We thank T Geiger, Y Roth, and S Gross (Boehringer Ingelheim) for their excellent experimental contributions. Medical writing and editorial assistance were provided
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. performed, designed experiments and analyzed results. A.W., C.B., N.R., L.P., F.G., F.R., and P.L.H. provided material. H.Y., 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.

FUNDING

This study was supported by Actions de Recherche Concertées 18/23-094 from the Communauté française de Belgique, by CDR grant J.0178.17 and PDR Grants T.0124.15 and T.0110.20 from the Fonds de la Recherche Scientifique-FNRS, by the Société Francophone du Diabète (Paris, France), by a grant from the European Foundation for the Study of Diabetes, by a grant 1912-03555 from the Leona M. & Harry B. Helmsley Charitable Trust, and by a grant from Boehringer Ingelheim Pharma GmbH & Co. KG, Biberach, Germany. P.G. is Research Director of the Fonds National de la Recherche Scientifique, Brussels. MB was supported by a fellowship from the ‘Fonds de Recherche Clinique’, Cliniques Universitaires Saint-Luc, UCLouvain, Brussels, Belgium. F.K. is holder of a fellowship from the
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 conflict of interest in connection with this manuscript.

PRIOR PRESENTATION

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

REFERENCES


FIGURE LEGENDS

Figure 1: Gliflozins increase glucosuria and induce an increase in the glucagonemia/insulinemia ratio that is correlated with a drop 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 on 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; 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 the 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 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, ***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 versus G7 or G2 +
dapagliflozin 1 µM: mean of 20–36 and 60–80 min versus mean of 40–56 min; G7 or G2

G7 or G2 + dapagliflozin 10 µM: mean of 60–80 and 108–128 min versus mean of 84–104 min; G7 and G2 versus G7 or G2 + arginine 10 mM: mean of 108–128 and 156–168 min versus mean of 132–148 min). *p<0.05 and **p<0.01, paired t-test for comparison between different conditions.

**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 comparison. (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 versus G10 + dapagliflozin or empagliflozin: mean of 20–36 and 76–108 min versus mean of 40–72 min; G0.5 versus G0.5 + dapagliflozin or empagliflozin: mean of 132–148 and 188–204 versus mean of 152–184 min; G10 versus G0.5 + sotagliflozin: mean of 20–36 and 92–108 min versus mean of 40–68 min; G0.5
versus G0.5 + sotagliflozin: mean of 148–164 and 200–240 versus mean of 168–196 min).
*p<0.05 and **p<0.01, paired t-test for comparison between different conditions.

**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 versus G4 + gliflozin: mean of 20–52 and 88–120 min versus mean of 56–84 min; G15 versus G15 + gliflozin: mean of 148–160 and 196–228 versus mean of 164–192 min). In panel C, the trend of glucagon secretion increase induced by sotagliflozin in G4 was not significant when comparing the mean of 20–52 min versus 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 versus G7 + vehicle or gliflozin: mean of 20–48 and 76–104 min versus mean of 52–72 min; G15 versus G15 + vehicle or gliflozin: mean of 120–140 and 168-196 versus mean of 144–164 min; G15 Dz versus G15 K30: mean of 212–220 min versus 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 islets 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 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).

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 from 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 (A) and Segerstolpe (B) data.

Figure 7: SGLT2 protein is highly expressed in the kidney but is 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 take up $^{14}$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 $\text{Na}^+$-dependent because it was prevented by replacement of $\text{Na}^+$ by choline. Data are means ± SE of 4 repetitions. (C) Validation of the SGLT2 antibody by Western blot. Membranes of HEK293 cells expressing (HEK293-hSGLT2, bands 3 and 4) or not (band 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 blots using the SGLT2 antibody. As expected, PNGaseF removed all glycosylation residues on SGLT2 and induced a shift of the molecular weight. Loading of the gel was controlled by Western blot for GAPDH. Although the loading of islet extract exceeded that of control tissues, no SGLT2 band was detected in islets. The blot 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 seen in normal HEK293 cells which were visualized by the labeling of their nuclei in blue by dapi. (E) Immunodetection of
SGLT2 on 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 labelled 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 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.
Main figs
Figure 2

Islet incubations

A

Pancreas perfusions

B

C

D

E

F

G7

G2

Dapa 1

Empa 1

Sota 1

G7

G7

Dapa 10

Dapa 10

Arg

G2

Insulin (ng/min/pancreas)

Glucagon (pg/min/pancreas)

Glucose [mM]

0.0

0.2

0.4

0.6

0.8

1.0

Arginine

KCl

Adrenaline

Empagliflozin

Dapagliflozin

Glucose [mM]

0

200

400

600

800

1000

1.0

0.5

1.5

2.0

Time (min)

Glucagon (pg/min/pancreas)

G2

Dapa 1

Empa 1

Sota 1

G7

G7

Dapa 10

Dapa 10

Arg

G2

Insulin (ng/min/pancreas)

Glucagon (pg/min/pancreas)

Glucose [mM]

0

100

200

300

400

500

600

0.0

0.1

0.2

0.3

1.0

0.5

1.0

1.5

2.0

Time (min)

Glucagon (pg/min/pancreas)

G2

Dapa 1

Empa 1

Sota 1

G7

G7

Dapa 10

Dapa 10

Arg

Dapa 1

Dapa 10

Arg

G7

G7

G2

Dapa 1

Dapa 10

Arg

G7

G7

G2

Dapa 1

Dapa 10

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Dapa 1

Dapa 10

Arg

G7

G7

G2

Dapa 1

Dapa 10

Arg

G7

G7

G2

Dapa 1

Dapa 10

Arg

G7

G7

G2

Dapa 1

Dapa 10

Arg

G7
Figure 3

A. Islet incubations

B. Islet perfusions

C. Islet perfusions

D. Islet perfusions

E. Islet perfusions

F. Islet perfusions

G. Islet perfusions

H. Islet perfusions

Numerical data and statistical significance are not explicitly mentioned in the diagram but can be inferred from the chart legends and error bars.
Figure 4

A. Dapa 1

B. Empa 1

C. Sota 1

D. Vehicle

E. Dapa 1

F. Empa 1

G. Sota 1

Islet perifusions

Journal Pre-proof
Figure 5

A. Rat Insulin

B. Rat Glucagon

C. Rat SGLT2

D. Mouse Insulin

E. Mouse Glucagon

F. Mouse SGLT1

G. Mouse SGLT2

H. Mouse GLUT1

I. Mouse GLUT2

J. Human Insulin

K. Human Glucagon

L. Human SGLT1

M. Human SGLT2

N. Human GLUT1

O. Human GLUT2
Figure 6

A

Enge

SLC2A isoforms male
SLC5A isoforms male
SLC2A isoforms female
SLC5A isoforms female
SLC5A2 male
SLC5A2 female

B

Segerstolpe

SLC2A isoforms normal
SLC2A isoforms T2D
SLC5A isoforms normal
SLC5A isoforms T2D
SLC5A2 normal
SLC5A2 T2D

C

Enge

SGLT1

D

Segerstolpe

SGLT1

SGLT2
Figure 7

A

B

C

D

E

F

GAPDH

hSGLT2

hSGLT2
deglycosylated

HEK293

HEK293-hSGLT2

Kidney

HEK293 Phlorizin

HEK293 Empagliflozin

HEK293-hSGLT2 Phlorizin

HEK293-hSGLT2 Empagliflozin

Choline Cl Buffer

EC50

Empagliflozin: 4.3nM

Phlorizin: 41 nM

cpm

log [conc] (M)

log [conc] (M)

1

2

3

4

5

6

7

1: HEK293

2: HEK293 + PNGaseF treated

3: HEK293-hSGLT2

4: HEK293-hSGLT2 + PNGaseF treated

5: Kidney

6: Kidney + PNGaseF treated

7: Human Islets

SLC5A2/RPLP0 (absolute ratio)

ND

NaCl Buffer

Mouse

Pancreas

Insulin

Glucagon

Somatostatin

SGLT2

Merged

Human

Rat

Mouse
Figure 8

The figure shows bar charts comparing the Gcg/Rplp0 (absolute ratio) at different glucose levels (11 and 25 mM) for Empagliflozin 10 and Dapagliflozin 10.

On the left side:
- Glucose 11 mM: Empagliflozin 10 (light blue), Dapagliflozin 10 (dark blue)
- Glucose 25 mM: Empagliflozin 10 (light blue), Dapagliflozin 10 (dark blue)

On the right side:
- Glucose 5 mM: Empagliflozin 10 (light blue), Dapagliflozin 10 (dark blue)
- Glucose 15 mM: Empagliflozin 10 (light blue), Dapagliflozin 10 (dark blue)
Highlights

- Gliflozins (SGLT2 and SGLT1/2 inhibitors) increase plasma glucagon/insulin ratio
- SGLT2 is not expressed in rodent and human pancreatic α- and β-cells.
- SGLT1 is however expressed in human α-cells
- SGLT2 and SGLT1/2 inhibitors do not directly affect glucagon and insulin secretion
Declaration of interests

☐ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☒ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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 conflict of interest in connection with this manuscript