



The Role of Glucagon in the Acute Therapeutic Effects of SGLT2 Inhibition

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Sodium–glucose cotransporter 2 inhibitors (SGLT2i) effectively lower plasma glucose (PG) concentration in patients with type 2 diabetes, but studies have suggested that circulating glucagon concentrations and endogenous glucose production (EGP) are increased by SGLT2i, possibly compromising their glucose-lowering ability. To tease out whether and how glucagon may influence the glucose-lowering effect of SGLT2 inhibition, we subjected 12 patients with type 2 diabetes to a randomized, placebo-controlled, double-blinded, crossover, double-dummy study comprising, on 4 separate days, a liquid mixed-meal test preceded by single-dose administration of either 1) placebo, 2) the SGLT2i empagliflozin (25 mg), 3) the glucagon receptor antagonist LY2409021 (300 mg), or 4) the combination empagliflozin + LY2409021. Empagliflozin and LY2409021 individually lowered fasting PG compared with placebo, and the combination further decreased fasting PG. Previous findings of increased glucagon concentrations and EGP during acute administration of SGLT2i were not replicated in this study. Empagliflozin reduced postprandial PG through increased urinary glucose excretion. LY2409021 reduced EGP significantly but gave rise to a paradoxical increase in postprandial PG excursion, which was annulled by empagliflozin during their combination (empagliflozin + LY2409021). In conclusion, our findings do not support that an SGLT2i-induced glucagonotropic effect is of importance for the glucose-lowering property of SGLT2 inhibition.

Sodium–glucose cotransporter 2 inhibitors (SGLT2i) are glucose-lowering agents that lower plasma glucose (PG) concentrations by increasing urinary glucose excretion (1,2). SGLT2 is highly expressed in the proximal renal tubule and accounts for the majority of reabsorption of glucose filtered through the glomeruli (3). In type 2 diabetes, SGLT2i improve glycemic control, with a low rate of hypoglycemia and body weight loss as an additional advantage (4–6). Another attractive feature of SGLT2i is a reduced risk of major adverse cardiovascular events in high-risk patients (7,8). SGLT2i have been reported to increase glucagon concentrations and augment endogenous glucose production (EGP) in mice (9) as well as in individuals with type 2 diabetes (10–12). The reported glucagonotropic effect of SGLT2i may reflect a compensatory mechanism caused by declining blood glucose or glucosuria and/or could be a direct effect of the SGLT2i on the pancreatic α -cells (9). In support of the latter, expression of SGLT2 in the pancreatic α -cells has been described (9,13,14). However, conflicting data are emerging, and the direct effect of SGLT2i on 1α -cells is under debate (15,16).

Patients with type 2 diabetes are characterized by fasting hyperglucagonemia, and inappropriate suppression of glucagon together with increased EGP after meal intake (17,18) have previously been linked to postprandial hyperglycemia in type 2 diabetes (19,20). Thus, further augmentation of glucagon levels during SGLT2i treatment may attenuate the

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glucose-lowering effect of this drug class. Therefore, the concept of combining SGLT2i with agents counteracting glucagon-mediated effects on EGP has received much attention (18,21). Clinical studies investigating combination therapy with glucagon-like peptide 1 (GLP-1) receptor agonist (lower glucagon levels and reduced EGP) and SGLT2i in patients with inadequately controlled type 2 diabetes have shown additive glucose-lowering effects (22,23). Antagonization of the glucagon receptor represents another strategy to reduce hyperglucagonemia-associated hyperglycemia in type 2 diabetes (18,24–26). However, the clinical development of several glucagon receptor antagonists (GRAs) has been discontinued because of side effects, including hepatic steatosis and increased concentrations of total cholesterol, LDL cholesterol, and hepatic transaminases (26–28), combined with concerns about α -cell hyperplasia in preclinical studies (26). Here, we tested the hypothesis that a glucagonotropic effect of SGLT2i would affect the glucose-lowering effect of SGLT2i in type 2 diabetes by applying single doses of the SGLT2i empagliflozin with and without the GRA LY2409021 in a randomized, placebo-controlled, double-blinded, crossover study involving four liquid mixed-meal tests (MMTs) in patients with type 2 diabetes.

RESEARCH DESIGN AND METHODS

Ethical Approval

This study protocol was approved by the ethical committee of The Capital Region of Denmark (H-15018701), and the study was conducted in accordance with standards of good clinical practice, the Declaration of Helsinki, and all applicable local regulations.

Participants

Patients with type 2 diabetes were recruited from the diabetes outpatient clinic at the Department of Medicine, Gentofte Hospital, or by advertising. Thirteen patients were included (12 completed) on the basis of the following inclusion criteria: Caucasians >30 years of age with diet- and/or metformin-treated type 2 diabetes diagnosed according to the World Health Organization (29) for at least 3 months and a normal hemoglobin. The following exclusion criteria were used: inflammatory bowel disease, intestinal resections, nephropathy (serum creatinine above normal range and/or albuminuria), known or suspected liver disease (hepatic transaminases elevated more than two times the upper normal level), treatment with medicine that could not be paused for 12 h, loop diuretics or glucose-lowering medications other than metformin, pregnancy and/or breastfeeding, family history of pancreatic islet tumors, age >70 years, and chronic heart failure.

Study Medication

GRA LY2409021 was provided as a gift from Eli Lilly (Indianapolis, IN). In clinical trials, this selective, orally administered, competitive, small-molecule GRA has a median time for maximum drug concentration (T_{\max}) ranging

from 4 to 8 h and a mean half-life ($T_{1/2}$) ranging from 51 to 59 h (30). A dose of 300 mg at the start of the fast the evening before the experimental day was chosen to achieve near-maximal glucagon receptor antagonism and clear reduction of EGP during the following study day (30,31). The SGLT2i empagliflozin has a T_{\max} of 1–2 h and a $T_{1/2}$ of ~12 h after single-dose administration of 25 mg, and it was therefore administered 2 h before meal ingestion (with 50 mL water) and expected to result in near-maximum inhibition during the mixed meal (1,32).

Study Design and Experimental Procedures

This study was a double-blinded, placebo-controlled, double-dummy, crossover study consisting of a screening visit and 4 experimental days performed in randomized order: 1) placebo + placebo, 2) SGLT2i (empagliflozin 25 mg) + placebo, 3) GRA (LY2409021 300 mg) + placebo, and 4) GRA (LY2409021 300 mg) + SGLT2i (empagliflozin 25 mg). Days were separated by a minimum of 2 weeks to ensure washout of LY2409021. All experiments were performed at Gentofte Hospital, University of Copenhagen. Glucose-lowering treatment was paused for 1 week before each study day. Participants met in the morning after a 10-h fast (including liquids, medicine, and tobacco), and were placed in a hospital bed in a semirecumbent position. Two cannulas were inserted in the cubital veins: one for infusions and one in the contralateral arm for collection of arterialized blood samples using the heated hand technique (hand and forearm wrapped in heating pad at ~45°C). At time –120 min, empagliflozin/placebo was ingested (with 50 mL water), and a primed constant infusion of stable isotopes (Cambridge Isotope Laboratories, Tewksbury, MA) dissolved in saline was initiated ([6,6-²H₂]glucose [priming dose 17.6 $\mu\text{mol} \times \text{kg}^{-1}$ fasting PG (FPG) / 5 and constant infusion of 0.6 $\mu\text{mol} \times \text{kg}^{-1} \times \text{min}^{-1}$] and [1,1,2,3,3-D₅]glycerol [priming dose 2 $\mu\text{mol} \times \text{kg}^{-1}$ and constant infusion of 0.1 $\mu\text{mol} \times \text{kg}^{-1} \times \text{min}^{-1}$]). At time 0 min, participants ingested a standardized liquid MMT (47.2 g anhydrous glucose, 15.2 g whey protein powder, and 14.1 g grapeseed oil mixed in 150 mL water; 200 mL, 394 kcal, 50 proportion of energy (%E) carbohydrates, 15%E protein, and 35%E fat) over a period of 10 min. Acetaminophen (1.5 g) was added to the liquid meal for evaluation of gastric emptying rate (33,34) and 2.8 g [U-¹³C₆]glucose for tracing the orally ingested glucose (double-tracer technique) (35,36). After the 4-h MMT, participants were served an ad libitum meal of pasta Bolognese (energy content per 100 g: 147 kcal, 17.4 g carbohydrates, 5.6 g protein, 5.9 g fat) and were told to eat until pleasantly satiated.

Data Collection

Before initiation of experimental procedures, participants were instructed to empty their urinary bladder and again at time 240 min; urine volume was registered, and samples were collected and stored at –20°C for subsequent measurements of glucose and tracer concentrations. Blood

samples were drawn at time -120 , -45 , -30 , -15 , 0 , 10 , 20 , 30 , 50 , 70 , 90 , 120 , 150 , and 240 min. For bedside measurements of PG, blood was collected in sodium fluoride-coated tubes and centrifuged immediately (30 s, room temperature, 7,500g). Blood for the analysis of glucagon and isotope enrichments was collected in prechilled EDTA tubes with dipeptidyl peptidase 4 inhibitor (valine pyrrolidide 0.01 mmol/L; gift from Novo Nordisk, Måløv, Denmark). Samples collected in lithium heparin tubes (acetaminophen) and dry tubes with serum separator gel and silica particles for clot activation (C-peptide) were left to coagulate (20 min, room temperature). All blood samples were centrifuged (15 min, 4°C, 2,900g) and stored afterward at -20°C (plasma) or -80°C (serum) until study completion and later analysis. Energy intake during the ad libitum meal was calculated by subtracting the amount of weighed leftovers from the amount of weighed food served. Resting energy expenditure and respiratory quotient were measured by indirect calorimetry with a tight face mask, measuring gas exchange breath by breath (CCM Express; MedGraphics Diagnostics, St. Paul, MN), for 12 min at baseline before the MMT and at time 30 min.

Laboratory Methods

PG was analyzed at bedside using the glucose oxidase method (Model 2300 Stat Plus and 2900 Biochemistry Analyzers; Yellow Springs Instruments, Yellow Springs, OH). Glucose and glycerol concentrations and plasma enrichments of $[6,6\text{-}^2\text{H}_2]$ glucose, $[1,1,2,3,3\text{-D}_5]$ glycerol, and $[\text{U-}^{13}\text{C}_6]$ glucose were measured by liquid chromatography-tandem mass spectrometry as previously described (37). Serum C-peptide was measured using a two-site sandwich immunoassay with direct chemiluminescent technology (ADVIA Centaur XP; Siemens Healthcare A/S, Ballerup, Denmark). Plasma acetaminophen analysis was based on amidase hydrolysis, oxidation, and linkage to tetrahydroquinoline, producing a color shift measured by reflectance photometry (670 nm) (Vitros 5.1 FS; Ortho-Clinical Diagnostics). Plasma glucagon was measured with an in-house radioimmunoassay (antibody 4305) directed against the COOH-terminal (38), which has been validated thoroughly as recently discussed (39).

Calculations and Statistical Analyses

Results are reported as mean \pm SEM unless otherwise stated. Area under the curve (AUC) was calculated using the trapezoidal rule. Statistical comparisons were made with a linear mixed model, with experimental day as the fixed effect and with an unstructured covariance pattern to account for correlation between repeated measurements in the same individual. Mixed-model analyses were performed with SAS Enterprise Guide 7.1 (SAS Institute, Cary, NC) (40). To reduce the risk of false positives as a result of multiple testing, all P values were adjusted (adj.) using the method of Benjamini and Hochberg (41) to control the false discovery rate (i.e., an adj. $P \leq 0.05$ means that the

reported significance is $\leq 5\%$ likely to be false positive). All participants included in the analyses completed all experimental days. Tracer isotope data are displayed as glucose R_a and R_d calculated from changes in glucose enrichment using Steele's one-compartment, fixed-volume (fixed pool fraction of $70 \text{ mL} \times \text{kg}^{-1}$), nonsteady-state model for stable isotopes (42). Insulin secretion rate (ISR) was calculated by deconvolution of C-peptide and C-peptide kinetics (43,44). HOMA insulin resistance was calculated from fasting C-peptide and glucose with HOMA calculator version 2.2.3 software (Diabetes Trials Unit, University of Oxford, <https://www.dtu.ox.ac.uk/homacalculator>).

Data and Resource Availability

The data sets generated and analyzed during the current study are not publicly available but are available from the corresponding author upon reasonable request.

RESULTS

Participant Characteristics

In total, 13 patients were randomized. Twelve patients with type 2 diabetes completed all 4 study days and were included in the study (Table 1). Eleven patients were treated with metformin only (well controlled on 0.5–2 g daily), and one patient was treated with diet only. One participant with a history of migraines dropped out after the 2nd experimental day (not included in analyses) because of a migraine after the 1st experimental day. This participant was replaced by another to ensure 12 completing participants.

Glucose Concentrations

FPG was lowered by both empagliflozin and LY2409021 compared with placebo (mean difference \pm SEM -1.0 ± 0.2 and -2.0 ± 0.2 mmol/L, respectively, $P \leq 0.0001$, adj. $P \leq 0.0006$), and combined FPG was lowered even further compared with placebo (-2.4 ± 0.2 mmol/L, $P \leq 0.0001$, adj. $P \leq 0.0006$) (Table 2 and Fig. 1A and B). The peak PG during the MMT was lowered significantly by empagliflozin, LY2409021, and the combination of the two (Table 2 and Fig. 1A). Empagliflozin and LY2409021 lowered peak PG similarly ($P = 0.526$), but the combination

Table 1—Participant characteristics

	Patients with type 2 diabetes
<i>N</i> (male/female)	12 (9/3)
Age (years)	59.5 \pm 5.8
BMI (kg/m ²)	30.3 \pm 5.6
HbA _{1c} (%)	6.5 \pm 2.7
HbA _{1c} (mmol/mol)	47.3 \pm 6.2
FPG (mmol/L)	8.1 \pm 0.9
HOMA of insulin resistance	2.09 \pm 1.0
Type 2 diabetes duration (years)	6.3 \pm 3.6

Data are mean \pm SD unless otherwise indicated.

Table 2—Summarized results				
	Placebo	SGLT2i	GRA	GRA + SGLT2i
Glucose				
Fasting plasma (mmol/L)	7.9 ± 0.3	6.9 ± 0.3**	5.8 ± 0.2***§§	5.4 ± 0.3***§§¶¶
Peak plasma (mmol/L)	13.1 ± 0.6	11.2 ± 0.4**	11.5 ± 0.5*	9.8 ± 0.6***§¶¶
AUC _{0–240 min} (mmol/L × min)	2,367 ± 125	2,009 ± 89**	2,155 ± 90	1,812 ± 94***§¶¶
bsAUC _{0–240 min} (mmol/L × min)	479 ± 66	357 ± 51*	752 ± 58***§§	511 ± 53§¶¶
Glucagon				
Fasting plasma (pmol/L)	6.8 ± 1.8	7.2 ± 1.2	25.8 ± 3.8*§	29.1 ± 3.3***§§
AUC _{0–240 min} (pmol/L × min)	2,079 ± 430	2,277 ± 288	6,250 ± 1,157*§	7,130 ± 752***§§
bsAUC _{0–240 min} (pmol/L × min)	459 ± 143	557 ± 167	70 ± 380	150 ± 300
C-peptide				
Fasting plasma (pmol/L)	786 ± 87	687 ± 77	559 ± 67**§	527 ± 57*§
AUC _{0–240 min} (nmol/L × min)	398 ± 25	342 ± 28*	395 ± 32	323 ± 26*¶
bsAUC _{0–240 min} (nmol/L × min)	209 ± 60	177 ± 47	261 ± 24*§	197 ± 18¶
ISR				
Fasting plasma (pmol/L)	2.15 ± 0.2	1.93 ± 0.2	1.55 ± 0.1**§	1.52 ± 0.1*§
AUC _{0–240 min} (nmol/L × min)	1,095 ± 56	954 ± 69	1,090 ± 75	860 ± 62*¶
bsAUC _{0–240 min} (nmol/L × min)	579 ± 58	490 ± 49	719 ± 71*§	495 ± 51¶
Acetaminophen				
Peak plasma (μmol/L)	89.9 ± 6	91.6 ± 6	90.8 ± 7	89.1 ± 6
AUC _{0–240 min} (mmol/L × min)	14.7 ± 1.1	15.1 ± 1.2	15.1 ± 1.0	14.9 ± 1.0
T _{max} (min)	103 ± 8	115 ± 8	103 ± 11	125 ± 15

Data are mean ± SEM. Summarized results of placebo, SGLT2i (25 mg empagliflozin), GRA (300 mg LY2409021), and the combination (GRA + SGLT2i) days. Data are summarized as AUC and bsAUC. Comparisons between days are performed with linear mixed models, and *P* values are corrected for multiple comparisons by false discovery rate. **P* ≤ 0.05 compared with placebo. ***P* ≤ 0.001 compared with placebo. §*P* ≤ 0.05 compared with SGLT2i. §§*P* ≤ 0.001 compared with SGLT2i. ¶*P* ≤ 0.05 compared with GRA. ¶¶*P* ≤ 0.001 compared with GRA.

(empagliflozin + LY2409021) lowered peak PG significantly more than each compound alone (vs. empagliflozin *P* = 0.007 [adj. *P* = 0.020], vs. LY2409021 *P* < 0.0001 [adj. *P* < 0.001]) (Table 2 and Fig. 1A). Time to peak of PG was 103 ± 12 min for placebo and 100 ± 11 min for empagliflozin and tended to increase with LY2409021 (118 ± 11 min) and decrease with empagliflozin + LY2409021 (91 ± 10 min); however, all differences were insignificant. Compared with placebo, AUC was significantly lowered by empagliflozin (*P* < 0.001, adj. *P* < 0.001) and less convincingly by LY2409021 (*P* = 0.037, adj. *P* = 0.079), but AUCs during LY2409021 and empagliflozin were similar (*P* = 0.767) (Table 2 and Fig. 1A and C). The combination of empagliflozin + LY2409021 reduced AUC even further (Fig. 1A and C). The baseline-subtracted AUC (bsAUC) was significantly lower with empagliflozin compared with placebo, whereas LY2409021 significantly increased bsAUC (Table 2 and Fig. 1D), but when the agents were combined, empagliflozin eliminated the rise in bsAUC observed with LY2409021 (Table 2 and Fig. 1D).

Plasma Glucose Kinetics and Urine Excretion

Empagliflozin alone (16.2 μmol × kg⁻¹ × min⁻¹) did not affect fasting EGP compared with placebo (16.8 μmol × kg⁻¹ × min⁻¹, *P* = 0.346), and adding empagliflozin to LY2409021 did not affect fasting EGP (13.6 μmol kg⁻¹ × min⁻¹) compared with LY2409021 alone (12.9 μmol kg⁻¹ × min⁻¹, *P* = 0.061, adj. *P* = 0.125) (Fig. 2E). In contrast, LY2409021 lowered fasting EGP compared with placebo and empagliflozin (both *P* < 0.0001, adj. *P* < 0.001), and

the combination of empagliflozin + LY2409021 reduced fasting EGP compared with placebo and empagliflozin alone (both *P* < 0.001, adj. *P* < 0.01) (Fig. 2E). Postprandially, total glucose R_a was only affected (and reduced) by LY2409021 alone or in combination with empagliflozin, whereas empagliflozin alone did not affect glucose R_a (Fig. 2A and B). Glucose R_d was not reduced by empagliflozin, but LY2409021 and the combination of the two agents reduced R_d of glucose compared with placebo (Fig. 2G and H). Glucose excreted in the urine (Fig. 2J) and urine volume (Fig. 2I) were markedly increased on both empagliflozin days compared with placebo. Participants excreted a mean of 125 mmol (22.5 g) of glucose during the 2-h basal fasting plus 4-h postprandial experimental period with empagliflozin compared with 1.7 mmol (0.3 g) glucose with placebo (Fig. 2J). Urine glucose excretion was lower on the combination day (17.5 g glucose) compared with empagliflozin alone (Fig. 2J). The EGP during the 240 min was not changed by empagliflozin alone compared with placebo (Fig. 2E and F), but EGP was reduced by LY2409021 and empagliflozin + LY2409021 compared with placebo. When adding empagliflozin to LY2409021, EGP was increased compared with LY2409021 alone (Fig. 2E and F). R_a of the oral glucose tracer was similar on the 4 treatment days (Fig. 2C and D).

Glucagon

Compared with placebo, empagliflozin did not change fasting or postprandial plasma glucagon concentrations (Table 2 and Fig. 3A and B). In contrast, LY2409021

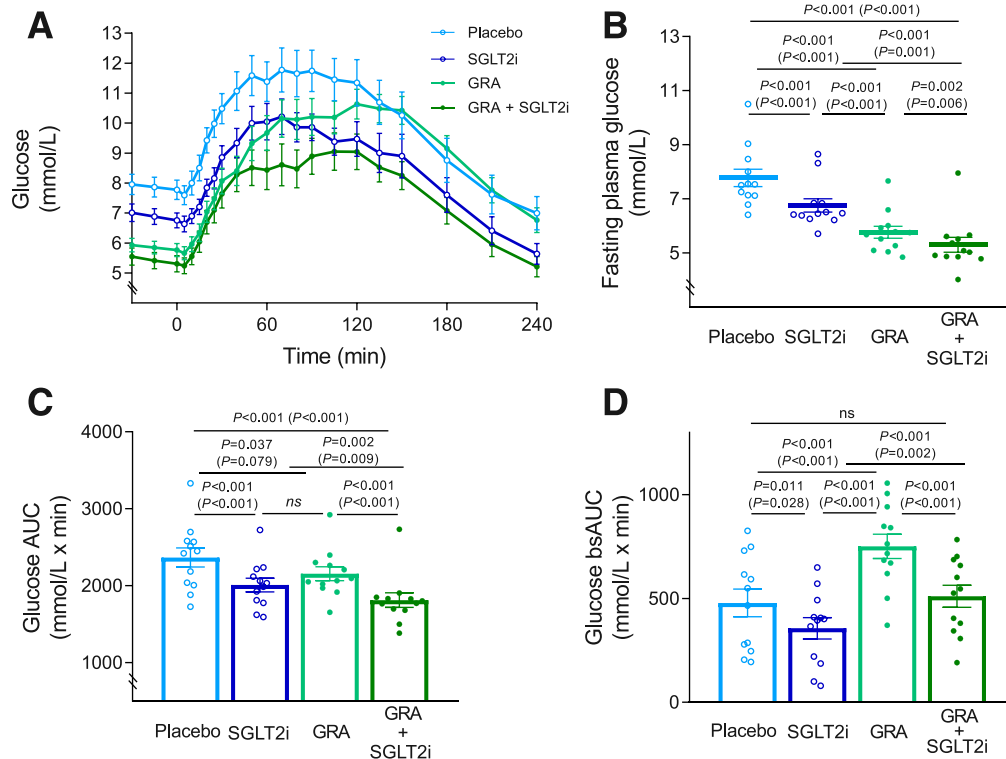


Figure 1—Fasting and postprandial glucose concentrations. PG concentrations during liquid MMT (A), fasting PG concentrations (B), AUC (C), and bsAUC (D) on days with placebo, the SGLT2i empagliflozin (25 mg), the GRA (300 mg LY2409021), and the combination (GRA + SGLT2i) in patients with type 2 diabetes ($N = 12$). Data are mean \pm SEM (symbols \pm error bars) (A) and mean \pm SEM (bars \pm error bars) with individual values (symbols) (B–D). Statistical comparisons were made with a linear mixed model, and P values illustrated are raw with P values adjusted for multiple comparisons by false discovery rate in parentheses. ns, not significant.

increased fasting plasma glucagon concentrations approximately threefold, and glucagon concentrations remained elevated throughout the MMT (Table 2 and Fig. 3A and B). Plasma glucagon concentrations during empagliflozin + LY2409021 were similar to glucagon concentrations during LY2409021 administration (Fig. 3A and B).

C-Peptide and ISR

LY2409021 and empagliflozin + LY2409021 significantly lowered fasting serum C-peptide concentrations compared with placebo and empagliflozin alone (Table 2 and Fig. 3C). Likewise, fasting ISR was reduced on all experimental days (insignificantly for empagliflozin) compared with placebo (Table 2). Postprandial excursions of C-peptide (AUC) were reduced compared with placebo by empagliflozin and empagliflozin + LY2409021 but were not affected by LY2409021 (Fig. 3D), whereas bsAUC of C-peptide was increased by LY2409021 compared with placebo and empagliflozin (Table 2 and Fig. 3D). Similarly, we observed a significant reduction of ISR AUC with empagliflozin + LY2409021 compared with placebo and LY2409021, and bsAUC of the ISR was increased with LY2409021 compared with placebo and empagliflozin (Table 2).

Glycerol Concentrations and Kinetics

During fasting conditions, empagliflozin + LY2409021 increased plasma glycerol concentration compared with

placebo ($P = 0.026$, adj. $P = 0.059$) and empagliflozin alone ($P = 0.0028$, adj. $P = 0.010$). LY2409021 ($P = 0.146$) and empagliflozin ($P = 0.652$) alone did not affect fasting glycerol concentration significantly (Fig. 4A). Interindividual variation was high (Fig. 4A), and postprandial excursions of glycerol (AUC) differed only between empagliflozin versus empagliflozin + LY2409021 ($P = 0.015$, adj. $P = 0.037$) and LY2409021 versus empagliflozin + LY2409021 ($P = 0.025$, adj. $P = 0.059$). Fasting glycerol R_a (Fig. 4B), representing the whole-body lipolytic rate, was higher with empagliflozin + LY2409021 compared with placebo ($P = 0.024$, adj. $P = 0.056$), empagliflozin ($P = 0.012$, adj. $P = 0.030$), and LY2409021 ($P = 0.006$, adj. $P = 0.018$). Likewise, AUC for glycerol R_a showed a tendency to be higher with empagliflozin + LY2409021 compared with placebo ($P = 0.133$) and was significantly higher compared with empagliflozin ($P = 0.006$, adj. $P = 0.016$) and LY2409021 ($P = 0.011$, adj. $P = 0.029$).

Gastric Emptying, Energy Intake, and Energy Expenditure

There were no differences in time to peak, peak acetaminophen concentration, or AUC of acetaminophen (proxy for gastric emptying) among any of the treatment days (Table 2 and Fig. 3E and F). Energy expenditure was measured before the MMT and 30 min after the MMT started, and we observed no differences in resting energy expenditure

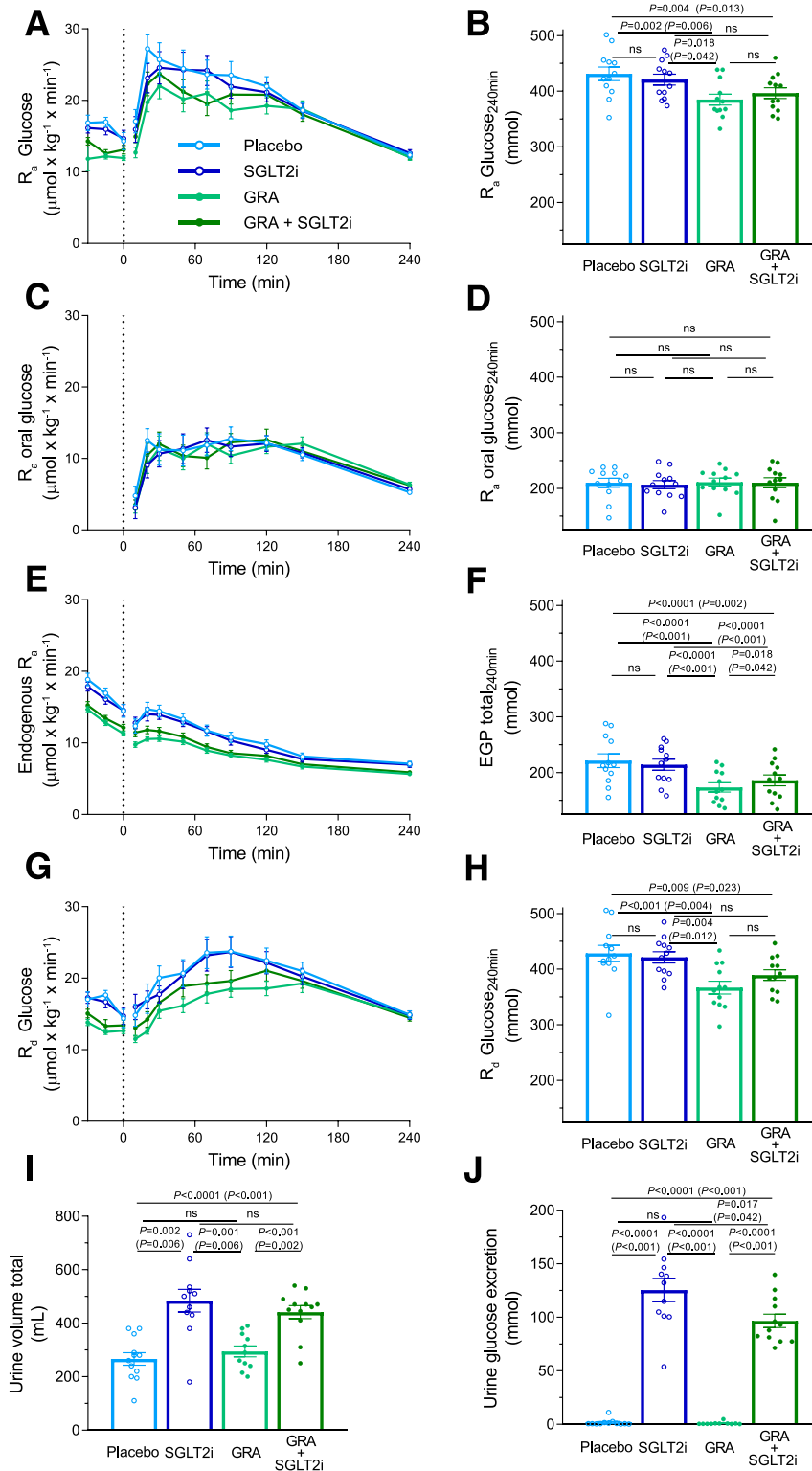


Figure 2—Glucose kinetics and urinary glucose excretion. R_a of total glucose (A), oral glucose (C), EGP (E), and R_d of glucose (G) with summarized mmol of glucose appearing or disappearing during the 240-min MMT (B, D, F, and H); urine volume (I); and urine glucose excretion (J) in patients with type 2 diabetes ($N = 12$). Data are mean \pm SEM (symbols \pm error bars) (A, C, E, and G) and mean \pm SEM (bars \pm error bars) with individual values (symbols) (B, D, F, and H–J). Statistical comparisons were made with a linear mixed model, and P values illustrated are raw with P values adjusted for multiple comparisons by false discovery rate in parentheses. ns, not significant.

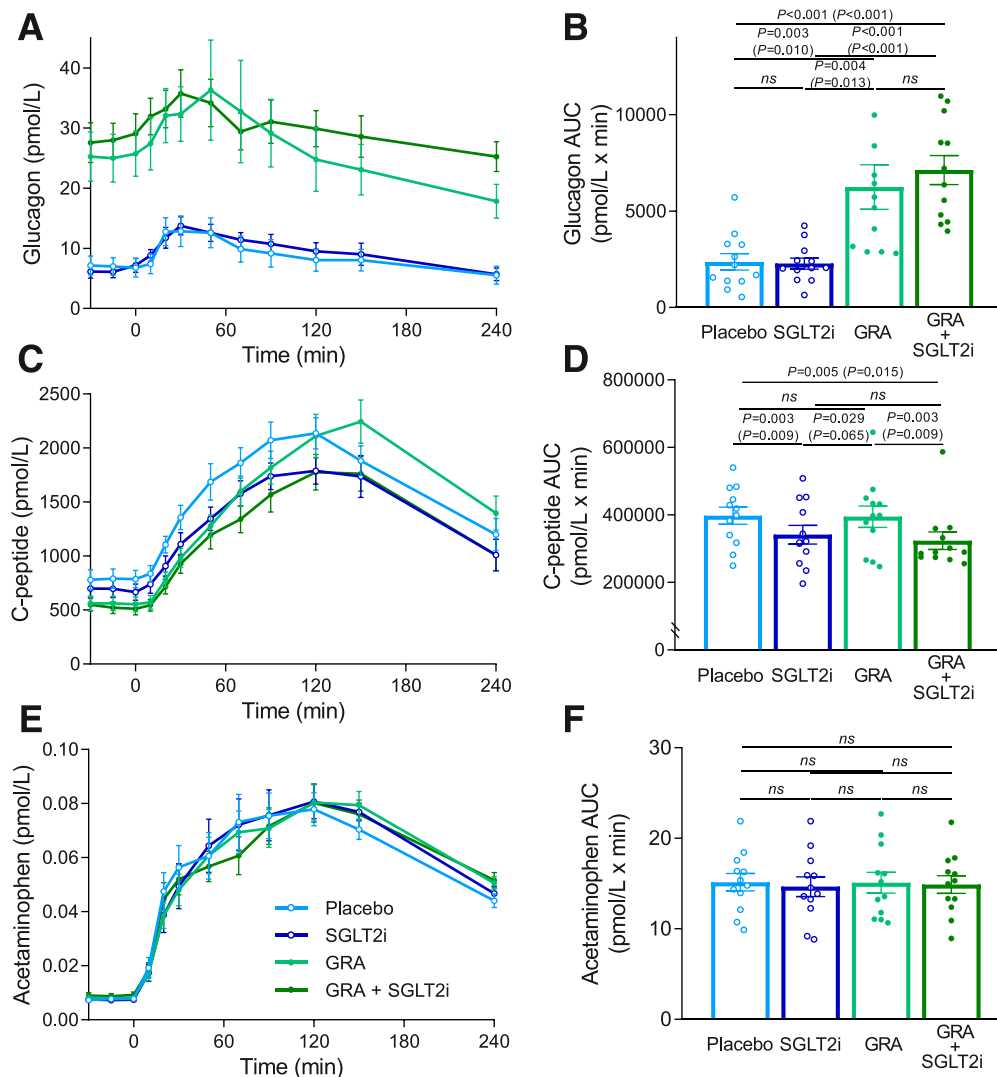


Figure 3—Glucagon, C-peptide, and acetaminophen. Concentrations of glucagon (A), C-peptide (C), and acetaminophen (E) before and during the MMT in patients with type 2 diabetes ($N = 12$); data are mean \pm SEM (symbols \pm error bars). Summarized results as AUCs; data are mean \pm SEM (bars \pm error bars) with individual values (symbols) (B, D, and F). Statistical comparisons were made with a linear mixed model and P values illustrated are raw with P values adjusted for multiple comparisons by false discovery rate in parentheses. ns, not significant.

or respiratory quotient among any of the treatment days (Supplementary Table 1). The amount of food consumed (energy intake) during the ad libitum meal after time point 240 min did not differ among the 4 days (Supplementary Table 1).

DISCUSSION

By subjecting patients with type 2 diabetes to MMTs preceded by single-dose administration of the SGLT2i empagliflozin, the GRA LY2409021, the combination empagliflozin + LY2409021, and double-dummy placebo, respectively, we report 1) no effects of empagliflozin on plasma glucagon concentrations or EGP, challenging previous findings; 2) modest and robust (subadditive) FPG-lowering effects of empagliflozin and LY2409021, respectively, through diverse mechanisms (empagliflozin through increased urinary glucose excretion and LY2409021 through reduced

EGP); and 3) a paradoxically larger increment in postprandial glucose excursions with LY2409021, which was eliminated when LY2409021 was administered together with empagliflozin. To our knowledge, this study is the first to evaluate the effect of an SGLT2i combined with a GRA in humans. We found no effects of empagliflozin on plasma glucagon and EGP. In a previous study that included patients with type 2 diabetes ($N = 66$), Ferrannini et al. (10) showed that a single dose of empagliflozin (25 mg) increased EGP. The authors explained this phenomenon as a result of increased glucagon concentrations and decreased insulin secretion after a MMT. In contrast, we observe no impact of empagliflozin (the same dose) on plasma glucagon concentrations or fasting or postprandial EGP. Nevertheless, we found that acute induction of glucosuria by empagliflozin leads to reductions in fasting and postprandial C-peptide concentrations, which is in

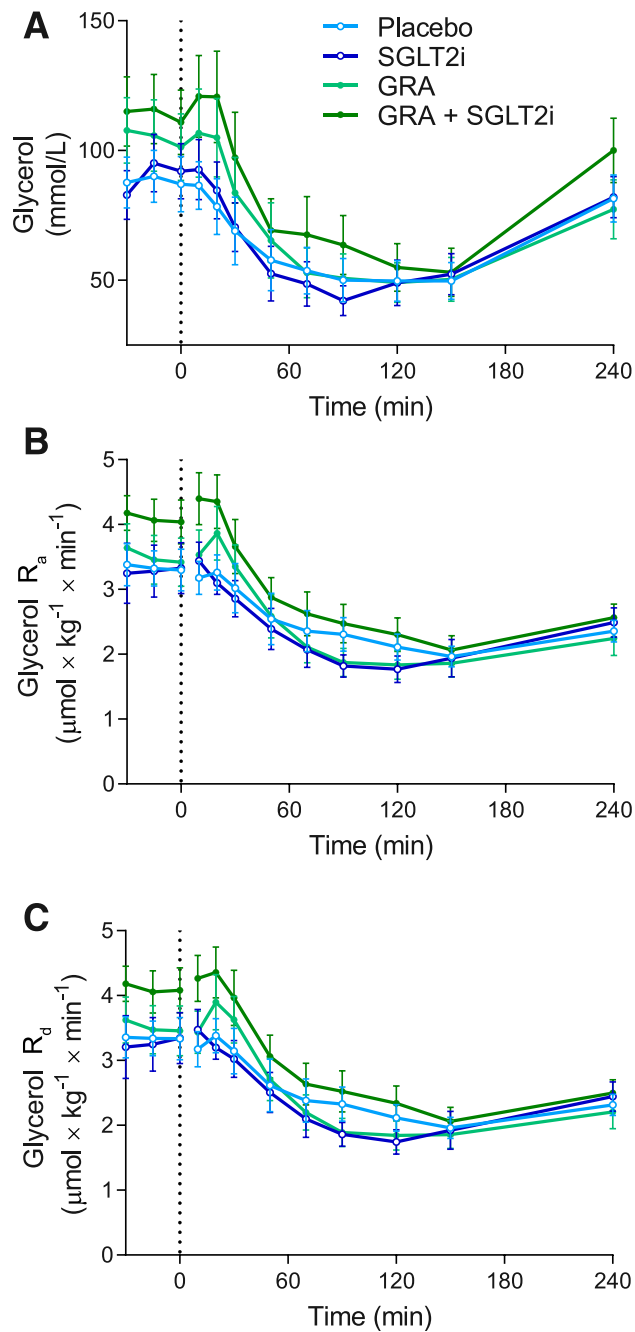


Figure 4—Glycerol concentrations and kinetics. Plasma glycerol concentrations during liquid MMT (A), R_a of glycerol (B), and R_d of glycerol (C) in patients with type 2 diabetes ($N = 12$). Data are mean \pm SEM.

agreement with the findings of Ferrannini et al. Differences between the studies may contribute to the explanation for the different observations related to glucagon and EGP. In our study, the labeled glucose in the liquid meal was dissolved in the total content of the meal, whereas the white bread-derived carbohydrates in the solid meal was imputed to calculate EGP by Ferrannini et al. In contrast to Ferrannini et al., who administered empagliflozin non-blinded 3.5 h before the meal test, we administered

empagliflozin 2 h before the MMT in a double-blinded fashion. The timing of empagliflozin dosing was based on pharmacokinetic properties of the compound demonstrating C_{max} for empagliflozin after 1–2 h (45), which is supported by the clear effects on urinary glucose excretion and the ensuing reductions in FPG and postprandial PG concentrations. Studying dapagliflozin-induced glucosuria in men with diabetes ($n = 12$ dapagliflozin, $n = 6$ placebo), Merovci et al. (11) found increased basal EGP (using a stable infusion of $[3-^3H]$ glucose) together with increased plasma glucagon concentrations 4 h after administration. Given the tendency of increased glucagon concentrations that we observed with empagliflozin after 2 h, we cannot completely exclude that we could have found a greater increase 3 or 4 h postdose as in the abovementioned studies. Thus, our data do not reproduce the findings of Ferrannini et al. and Merovci et al. with regard to increased glucagon concentrations and EGP after single doses of empagliflozin and dapagliflozin, respectively. In fact, we observed that EGP is larger with empagliflozin + LY2409021 compared with LY2409021 alone, suggesting that glucagon cannot explain the difference in EGP (glucagon receptors are blocked). We cannot rule out problems of sample size and type II errors with the limited number of participants ($N = 12$) in our study. However, a few other studies have reported similar glucagon data to ours with glucagon measured by Mercodia ELISA (46,47). The latter studies and ours have reported lower glucagon concentrations compared with those of Merovci et al. and Ferrannini et al., suggesting that differences in glucagon assays used may explain some of the differences in absolute concentrations of glucagon measured. The radioimmunoassay used in this study has been validated against mass spectrophotometry (39). Differences in glucagon assays might possibly explain a difference between ours and findings by others if, for example, SGLT2 inhibition by some unknown mechanism increases glucagon-like peptides and/or glucagon precursor sequences but not glucagon (33–61) itself. Increases in glucagon concentrations after SGLT2i treatment have been explained by direct stimulation of SGLT2 on α -cells, but recent data demonstrated large variability in SGLT2i effect on glucagon secretion in human islets (48), and direct effects on α -cells are still debated (15,16). In light of these new findings, it may not be surprising that empagliflozin did not increase glucagon concentrations in this study. Moreover, varying baseline glucose levels may play a role in glucagon secretion (49), and a higher glucagon-to-insulin ratio seems to be associated with poor glycemic control (50). New data have indicated that glucagon concentrations during acute SGLT2i treatment are mainly mediated through glycemic changes and that a direct effect on α -cells is of less importance (46); this is in line with higher (insignificant) fasting glucagon concentrations with LY2409021 + empagliflozin, where glucose concentration is lower compared with LY2409021. Previous studies indicated that longer-term treatment with empagliflozin and dapagliflozin may blunt the suggested increases in EGP and glucagon

concentrations (10,11); however, we were able to look at the acute effects of single doses only. Given the phenotype of our participants with type 2 diabetes (high total glucose), a longer tracer equilibrium period (e.g., of 180 min) might have been desirable during baseline.

We found no rise in EGP and glucagon concentrations with empagliflozin in this study but, nevertheless, observed a clinically relevant, subadditive reduction of FPG with the combination of empagliflozin + LY2409021 compared with the effects of LY2409021 and empagliflozin alone. Glucagon receptor antagonism is particularly efficient in lowering fasting and premeal glucose concentrations (51–54). With the double-tracer technique, we confirmed that FPG is reduced with LY2409021 as a result of reduction of fasting EGP. Empagliflozin and other SGLT2i exert their glucose-lowering effect by inducing glucosuria. In normal physiology, ~180 g of glucose is filtered through the glomeruli during 24 h, but all glucose is reabsorbed through SGLT-dependent mechanisms, so no glucose is excreted in healthy individuals (55). However, when glucose reabsorptive mechanisms are saturated above a threshold of ~11 mmol/L, glucose is excreted in a linear manner with increasing PG concentration (55). Patients with type 2 diabetes have increased expression and activity of SGLT2 (56), and thus, inhibition of renal glucose reabsorption with a single dose of 25 mg empagliflozin has previously been shown to result in a glucose excretion of 60–90 g per 24 h in these patients (57,58). This is in line with our results; we observed 22.5 g glucose excreted during the 6-h experimental day (fasting and prandial conditions). During the empagliflozin + LY2409021 day, we observed lower urinary excretion of glucose (17.5 g glucose per 6 h), which we ascribe to the lower PG concentrations on that day.

We report a paradoxically large increment in postprandial glucose excursions with LY2409021. Previous studies have been inconsistent in the effect of GRA on postprandial glucose excursions (51,52). We believe that this can be explained by cross-reactivity and inhibition of the glucose-dependent insulinotropic polypeptide (GIP) and GLP-1 receptor because of the high theoretical concentration of LY2409021 achieved with the dose of 300 mg. We have investigated the *in vitro* inhibitory effect of LY2409021 on the GIP and GLP-1 receptors (data not published), and on the basis of previous concentration-versus-time profiles (30), we estimate that with the dose of 300 mg, ~50–80% of GIP and GLP-1 receptor activity may be inhibited. Another possible explanation of the paradoxical increment in postprandial glucose excursions with LY2409021 could be diminished disappearance of glucose (lower R_d as seen in Fig. 2H) secondary to decreased glucose levels and, thereby, decreased glucose-mediated transport through GLUT-4 in muscle cells (59). SGLT2 inhibition lowers glucose excursions after an oral glucose tolerance test (58), and canagliflozin has shown a near 50% greater reduction of postmeal glucose compared with reduction of FPG (60) (possibly as a result of canagliflozin's low-

potency SGLT1 inhibition delaying intestinal glucose absorption as well) (4). We report a reduction of both AUC and bsAUC of glucose after a single dose of empagliflozin, which was due to increased urinary glucose excretion without any effect on EGP. Our data illustrate the efficient glucose-lowering combination of reduced fasting EGP with glucagon receptor antagonism and the postprandial glucose-lowering effect of an SGLT2i (reduction of urinary glucose excretion threshold).

SGLT2i have been associated with increased ketogenesis and a shift from glucose oxidation to hepatic fat oxidation. The mechanism has previously been explained by the possible connection to increased glucagon concentrations; however, recently, SGLT2i-induced ketosis has been shown to be independent of glucagon and insulin, suggesting that a complementary ketogenic factor is involved in SGLT2i-induced ketosis (61). The lack of increased glucagon concentrations with empagliflozin and the limited effect of glucagon on the glucose-lowering effect of empagliflozin in the current study support this hypothesis. There is no evidence that glucagon affects peripheral lipolysis in human physiology (62), but SGLT2i increases the concentration of nonesterified fatty acids (61). Here, we report no significant effect of LY2409021 and empagliflozin on the fasting glycerol concentration, but the combination of the two treatments increased fasting glycerol and postprandial AUC. The lack of significant results may represent type II errors. Insulin strongly inhibits peripheral lipolysis and thereby the supply of nonesterified fatty acids, and we believe that our glycerol results may reflect the lower insulin levels with empagliflozin + LY2409021 (lower insulin-mediated inhibition of lipolysis).

In the current study, we had a unique opportunity, as an exploratory, secondary end point, to investigate the combined effects of single dosing of a GRA and an SGLT2i on food intake and energy expenditure. SGLT2i promote a significant body weight loss through sustained glucose (energy) loss through the urine (4). SGLT2i-induced body weight loss could alternatively be caused by increased energy expenditure or decreased energy intake, but longer-term clinical trials with canagliflozin (52-week phase III) showed an initial period of body weight loss followed by a body weight plateau because of a compensatory increase in energy intake to match the calories lost in urine (63) without any effects on energy expenditure (10,63). Exogenous glucagon has been shown to decrease food intake by inducing satiety (64), but the effects of GRA on food intake and energy expenditure have not previously been reported. Here, we report no differences in *ad libitum* food intake after a single dose of LY2409021 and empagliflozin, individually or combined. Moreover, we confirmed previous findings of no effects on energy expenditure during acute administration of an SGLT2i (10,63).

In conclusion, in contrast to previous studies, we found that a single (clinically recommended) dose of empagliflozin increased neither glucagon concentrations nor EGP

in patients with type 2 diabetes. Nevertheless, we found that the combination of the SGLT2i empagliflozin and the GRA LY2409021 reduced FPG beyond their individual capacity in patients with type 2 diabetes. Glucagon receptor antagonism resulted in a paradoxically increased increment in postprandial baseline-corrected glucose excursions, which was eliminated by the empagliflozin-induced glucosuria. Taken together, our findings suggest that circulating glucagon levels do not limit the glucose-lowering effect of a single dose of empagliflozin in patients with type 2 diabetes.

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Author Contributions. S.H., A.L., F.K.K., and T.V. designed the study, wrote the study protocol, and wrote the manuscript. S.H., E.N.-H., and H.M. performed the study. G.v.H. and J.J.H. generated and interpreted data. All authors critically edited the manuscript and approved the final version. S.H. and T.V. are the guarantors of this work and, as such, had 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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