Am J Physiol Endocrinol Metab 315: E904–E911, 2018.
First published August 14, 2018; doi:10.1152/ajpendo.00233.2018.

RESEARCH ARTICLE

Endogenous glucose production after sequential meals in humans: evidence for more prolonged suppression after ingestion of a second meal

Teddy Ang, Greg M. Kowalski, and Clinton R. Bruce

Institute for Physical Activity and Nutrition, School of Exercise and Nutrition Sciences, Deakin University, Geelong, Australia

Submitted 12 June 2018; accepted in final form 13 August 2018

Ang T, Kowalski GM, Bruce CR. Endogenous glucose production after sequential meals in humans: evidence for more prolonged suppression after ingestion of a second meal. *Am J Physiol Endocrinol Metab* 315: E904–E911, 2018. First published August 14, 2018; doi:10.1152/ajpendo.00233.2018.—Single-meal studies have shown that carbohydrate ingestion causes rapid and persistent suppression of endogenous glucose production (EGP). However, little is known about the regulation of EGP under real-life eating patterns in which multiple carbohydrate-containing meals are consumed throughout the day. Therefore, we aimed to characterize the regulation of EGP in response to sequential meals, specifically during the breakfast-lunch transition. Nine healthy individuals (5 men, 4 women; 32 ± 2 yr; 25.0 ± 1.4 kg/m^2) ingested two identical mixed meals, each containing 25 g of glucose, separated by 4 h, and EGP was determined by the variable infusion tracer-clamp approach. EGP was rapidly suppressed after both meals, with the pattern and magnitude of suppression being similar over the initial 75-min postmeal period. However, EGP suppression was more transient after breakfast compared with lunch, with EGP returning to basal rates 3 h after breakfast. In contrast, EGP remained in a suppressed state for the entire 4-h postlunch period. This occurred despite each meal eliciting similar plasma glucose and insulin responses. However, there was greater suppression of plasma glucagon levels after lunch, likely contributing to this response. These findings highlight the potential for distinct regulation of EGP with each meal of the day and suggest that EGP may be in a suppressed state for much of the day, since EGP did not return to basal rates even after a lunch meal containing a modest amount of carbohydrate.

endogenous glucose production; glucagon; glycemic control; insulin; liver

INTRODUCTION

The liver is a key organ involved in regulating blood glucose levels. In addition to being an important site of glucose uptake and storage, the liver is the major site of endogenous glucose production (EGP), accounting for ~90% of EGP under fasting conditions (8). EGP is particularly sensitive to hormonal and metabolic cues, with rapid alterations occurring in response to relatively small changes in circulating insulin, glucagon, and glucose (8, 34). Indeed, the increase in plasma glucose and insulin as well as the suppression of glucagon following carbohydrate ingestion cause rapid suppression of EGP (2, 3, 5, 19, 25, 26, 28, 30), which helps accommodate the entry of meal-derived glucose into the circulation, thus preventing excessive glycemic excursions.

In addition to rapid suppression, EGP is persistently suppressed after carbohydrate ingestion (2, 3, 5, 19, 25, 26, 28, 30). Indeed, ingestion of relatively small amounts of glucose (i.e., 25–35 g) either alone (15) or as part of a mixed meal (26) suppresses EGP for at least several hours, while larger glucose doses (≥75 g) can suppress EGP for 6–8 h (2, 3, 5, 28, 30). In the context of typical daily eating patterns in which carbohydrate-containing meals are regularly consumed, it is possible that EGP remains suppressed for much of the day since sequential meals are likely to be eaten before EGP returns to basal. Thus it is important to understand how EGP is regulated in response not only to a single mixed meal but also to multiple mixed meals ingested over the course of a day.

In a meal simulation model employed to mimic typical daily life (breakfast, lunch, and dinner containing 45–70 g of glucose per meal), it was predicted that EGP would only briefly return to basal levels early in the evening just before dinner, supporting the notion that EGP may in fact remain in a suppressed state for much of the day (9). While this simulated pattern of EGP suppression has yet to be experimentally validated (9), there is one report examining the EGP response to sequential oral glucose loads ingested 3 h apart (6). Consistent with the model of Dalla Man (9), it was reported that the suppression of EGP by the first glucose load was enhanced after ingestion of the subsequent glucose load (6). However, negative EGP rates were reported for most of the period after the second glucose load (6), which was almost certainly due to non-steady-state measurement errors resulting from changes in the tracer-to-tracee ratio associated with the constant glucose tracer infusion protocol employed in the study for the estimation of EGP (24, 33). Such non-steady-state errors can be overcome by employing a variable infusion approach whereby tracer infusion rates are altered in a way that mimics the anticipated changes in endogenous glucose concentrations to maintain near-constant tracer-to-tracee ratios over time. However, to the best of our knowledge, no attempts have been made to consecutively measure EGP across multiple mixed meals throughout a single day while employing the gold-standard tracer-to-tracee clamp approach to avoid erroneous negative EGP rates (24, 33). Attaining such experimental data is important as they will provide deeper insight into how EGP is regulated under normal daily feeding cycles.

Therefore, we aimed to characterize how EGP is regulated in healthy humans during the breakfast-lunch transition using meals of realistic size and macronutrient composition. Since we have previously shown that maximal EGP suppression

Address for reprint requests and other correspondence: C. R. Bruce, Inst. for Physical Activity and Nutrition, School of Exercise and Nutrition Sciences, Deakin Univ., 221 Burwood Hwy., Burwood, Victoria 3125, Australia (e-mail: clinton.bruce@deakin.edu.au).

occurs after ingestion of glucose loads as small as 25 g (15), mixed meals containing modest amounts of carbohydrate (25 g glucose, eggs, and cheese; similar to 2 slices of bread with an omelet) were used to demonstrate the magnitude and duration of EGP suppression following ingestion of sequential meals.

MATERIALS AND METHODS

Participants. Nine healthy individuals (5 men, 4 women) were studied. The participant characteristics are presented in Table 1. The Deakin University Human Research Ethics Committee approved the study, which was conducted in accordance with the Declaration of Helsinki. The purpose, nature, and potential risks were explained, and informed written consent was obtained.

Experimental design. Participants were studied after an overnight (~10 h) fast, consuming only water from 2130 on the evening before study. Strenuous exercise was avoided for 48 h before study. Upon participants' arrival at the laboratory at 0700, height and weight were recorded. After they rested in a supine position for 5 min, blood pressure was determined with an automated sphygmomanometer. A 22-gauge cannula was then inserted into a dorsal hand vein in a retrograde fashion, with the hand placed in a heated Perspex box at 50°C for arterialized blood sampling. A second cannula was inserted into a vein in the contralateral forearm for the infusion of [6,6-^{2}H]glucose (Cambridge Isotope Laboratories, Tewksbury, MA). A primed (33 μmol/kg administered over 5 min) continuous infusion of [6,6-^{2}H]glucose (0.33 $\mu mol^{-} \cdot kg^{-1} \cdot min^{-1}$) commenced at 0730. After the prime, [6,6-^{2}H]glucose was infused for 120 min (−120 to 0 min), with blood sampled at −30, −20, −10, and 0 min.

At 0930 (0 min), participants ingested breakfast (1,681 kJ; 25 g carbohydrate, 26 g protein, and 22 g fat) consisting of two eggs, 50 g of mozzarella cheese, and gelatin containing 25 g of glucose with [1-^{2}H]glucose (8% enrichment; Cambridge Isotope Laboratories). After meal ingestion, the cup containing residual gelatin was rinsed with 20 ml of warm water and the rinse solution was consumed. Blood was collected in EDTA-containing Vacutainers at 10, 20, 30, 40, 50, 60, 75, 90, 120, 150, 180, 210, and 240 min after meal ingestion. Additional blood was collected into BD P800 Vacutainers (Becton Dickinson) containing protease inhibitors including dipeptidyl peptidase-4 inhibitors for determination of glucagon and active glucagon-like peptide-1 (GLP-1; 7–36 amide) at 0, 10, 20, 30, 60, 90, 120, 180, and 240 min.

At 1330 (240 min), participants ingested a second meal (lunch) identical to the first, with the only difference being that the gelatin contained [U-^{13}C]glucose (8% enrichment; Cambridge Isotope Laboratories). A small aliquot of the gelatin from each meal was collected to determine the enrichment of [1-^{2}H]glucose and [U-^{13}C]glucose. The schedule for blood sampling was the same as after breakfast. Blood samples were immediately placed on ice and later spun in a centrifuge at 4,400 rpm for 10 min at 4°C, and plasma was stored at −80°C.

Table 1. *Participant characteristics*

Participants, *n*	9 (5 men, 4 women)
Age, yr	32 ± 2
Body mass, kg	72.1 ± 3.8
Height, m	1.70 ± 0.04
BMI, kg/m^2	25.0 ± 1.4
SBP, mmHg	122 ± 3
DBP, mmHg	74 ± 2
Fasting plasma glucose, mM	4.6 ± 0.2
Fasting plasma insulin, pM	23.5 ± 5.2
Fasting plasma glucagon, pM	8.5 ± 1.0
Fasting plasma FFA, mM	0.21 ± 0.04
Fasting plasma triglycerides. mM	0.8 ± 0.1

Data are means ± SE. BMI, body mass index; DBP, diastolic blood pressure; FFA, free fatty acids; SBP, systolic blood pressure.

Table 2. *Infusion profile of [6,6-^{2}H]glucose*

Breakfast		Lunch	
Time, min	Infusion rate, % of basal	Time, min	Infusion rate, % of basal
0–10	75	240–250	65
10–20	65	250–260	55
20–30	55	260–330	50
30–75	50	330–360	55
75–90	80	360–420	60
90–120	85	420–450	70
120–150	90	450–480	80
150–240	100		

Rates are presented as % of basal [6,6-^{2}H]glucose infusion rate, which was 0.33 $\mu mol \cdot kg^{-1} \cdot min^{-1}$.

After the first bite of each meal, the infusion rate of [6,6-^{2}H]glucose was altered to mimic the anticipated changes in endogenous glucose concentration during the postprandial period. This variable infusion dual-tracer approach uses the tracer-to-tracee clamp to maintain a near-constant ratio between [6,6-^{2}H]glucose and endogenous glucose concentrations, thereby minimizing non-steady-state error (24, 33). Initially, the same [6,6-^{2}H]glucose infusion profile was used for each meal based on published infusion rates where we measured EGP after ingestion of a 25-g glucose load (15). However, data from the first three participants revealed a slight but consistent drop in the tracer-to-tracee ratio at 75 min after breakfast, whereas the tracer-to-tracee ratio remained steady after lunch. The infusion profile was therefore optimized by increasing the [6,6-^{2}H]glucose infusion rate between 75 and 240 min to achieve a near-constant tracer-to-tracee ratio during the postbreakfast period (Table 2).

Plasma analyses. Glucose was determined with the glucose oxidase method. Commercially available ELISA kits were used to determine insulin (ALPCO, Salem, NH), C-peptide (Millipore, Billerica, MA), glucagon (Mercodia, Uppsala, Sweden), and active GLP-1 (7–36 amide; ALPCO). Free fatty acids (FFA; Wako Chemicals, Richmond, VA) and triglycerides (TAG; Roche Diagnostics, Basel, Switzerland) were determined with commercially available kits. Insulin secretion rates were calculated via C-peptide deconvolution with ISEC (Insulin SECretion) software (12).

Gas chromatography-mass spectrometry. Plasma glucose tracer enrichment was analyzed with the glucose methyloxime pentapropionate derivative (1). Plasma (10 μl) was deproteinated by the addition of 100 μl of ice-cold analytical-grade methanol, followed by vortexing and centrifugation (4°C for 10 min at 13,000 rpm). The supernatant was transferred into a glass insert and dried with a speed vacuum (Labconco). Samples were derivatized by the addition of 50 μl of methoxyamine (Sigma-Aldrich, St. Louis, MO) in pyridine (20 mg/ml; Sigma-Aldrich), followed by incubation at 90°C for 1 h. The second derivatization step was performed through the addition of 100 μl of propionic anhydride (Sigma-Aldrich) followed by incubation at 60°C for 30 min. After incubation, excess reagent was evaporated under speed vacuum, and the dry residue was resuspended in 100 μl of analytical-grade ethyl acetate for GC-MS analysis.

Glucose enrichment was determined with a 7890B Gas Chromatography System and a 5977B Mass Selective Detector (Agilent Technologies, Santa Clara, CA) via positive chemical ionization using methane as the reagent gas and helium as the carrier. The glucose methyloxime pentapropionate derivative was analyzed by monitoring the molecular ions of 416–422 mass-to-charge ratio (*m/z*) (M0–M+6), with major ions of interest corresponding to M0 (naturally occurring glucose; 416 *m/z*), M+1 ([1-^{2}H]glucose; 417 *m/z*), M+2 ([6,6-^{2}H]glucose; 418 *m/z*), and M+6 ([U-^{13}C]glucose, 422 *m/z*). M+3 (419 *m/z*) isotopomers were also measured to

account for recycling of [U-^{13}C]glucose. Selective ion monitoring was performed with a 10-ms dwell time for each ion. Sample (0.5 μl) was injected with a 20-to-1 split ratio. Ion abundances were determined with the Mass Hunter Workstation (Agilent Technologies). The raw isotopomer data were corrected (mole percent excess) for natural isotopic background abundance skew with the matrix method (16).

Calculations. The concentrations of endogenous glucose as well as the three glucose tracers ([6,6-^{2}H]glucose, [1-^{2}H]glucose, and [U-^{13}C]glucose) were determined as previously outlined (24, 31). With this data, the glucose components G_{nat} (concentration of natural glucose in plasma from ingested and endogenously produced glucose), $G_{6,6-2H}$ (concentration of plasma [6,6-^{2}H]glucose), G_{1-2H} (concentration of plasma [1-^{2}H]glucose), and G_{13C} (concentration of plasma [U-^{13}C]glucose) were calculated:

$$G_{nat} = G\frac{1}{1 + E_{6,6\text{-}2H} + E_{1\text{-}2H} + E_{13C}}$$

$$G_{6,6\text{-}2H} = G\frac{E_{6,6\text{-}2H}}{1 + E_{6,6\text{-}2H} + E_{1\text{-}2H} + E_{13C}}$$

$$G_{1\text{-}2H} = G\frac{E_{1\text{-}2H}}{1 + E_{6,6\text{-}2H} + E_{1\text{-}2H} + E_{13C}}$$

$$G_{13C} = G\frac{E_{13C}}{1 + E_{6,6\text{-}2H} + E_{1\text{-}2H} + E_{13C}}$$

where G is the plasma glucose concentration and $E_{6,6-2H}$, E_{1-2H}, and E_{13C} are the fractional enrichment of [6,6-^{2}H]glucose, [1-^{2}H]glucose, and [U-^{13}C]glucose in the plasma, respectively. It is important to note that metabolism of [U-^{13}C]glucose produces M+2 and M+3 isotopomers via mitochondrial gluconeogenesis. Upon [U-^{13}C]glucose-derived 3-carbon precursor (lactate, pyruvate, alanine) entry into the gluconeogenic pathway, there is near-equilibrium exchange between oxaloacetate, malate, and fumarate that causes carbon scrambling (35). Therefore, it was assumed that M+2 and M+3 glucose isotopomers are produced in similar quantities from [U-^{13}C]glucose (17, 35). Accordingly, M+3 glucose enrichment (as a surrogate for recycled M+2) was subtracted from total M+2 enrichment to account for the contribution of [U-^{13}C]glucose-derived M+2, thus reflecting actual [6,6-^{2}H]glucose enrichment (i.e., $E_{6,6-2H}$). The concentration of unlabeled glucose derived from endogenous sources (G_{end}) was calculated by subtracting the contribution of ingested natural glucose (which is proportional to G_{1-2H} and G_{13C} as measured by the enrichment of [1-^{2}H]glucose and [U-^{13}C]glucose in breakfast and lunch, respectively, $E_{breakfast}$ and E_{lunch}) from the total glucose concentration (i.e., G_{nat}). After breakfast, G_{end} was determined by the following equation:

$$G_{end} = G_{nat} - \frac{G_{1\text{-}2H}}{E_{breakfast}}$$

After lunch, G_{end} was determined by the following calculation taking into account the contribution of glucose derived from breakfast:

$$G_{end} = G_{nat} - \left[\left(\frac{G_{1\text{-}2H}}{E_{breakfast}}\right) + \left(\frac{G_{13C}}{E_{lunch}}\right)\right]$$

Steele's non-steady-state single-compartment model was used to calculate EGP (see Ref. 35):

$$EGP = \frac{Inf - [pV(G_{end1} + G_{end2})/2 \times (TTR_2 - TTR_1)/(t_2 - t_1)]}{(TTR_2 - TTR_1)/2}$$

where G_{end1} and G_{end2} are endogenous glucose concentrations at times t_1 and t_2, TTR_1 and TTR_2 are the ratios between $G_{6,6-2H}$ and G_{end} at times t_1 and t_2, and V is the glucose volume of distribution and p the pool fraction, assumed to equal 200 ml/kg and 0.65, respectively (24).

Percent suppression of EGP over the 4-h postprandial period following each meal was determined by normalizing the area below basal to the basal area:

$$\%\ \text{suppression of EGP} = \frac{\int_0^{240}[EGP(t) - EGP_b]dt}{EGP_b \times 240} \times 100$$

where EGP_b is the basal rate of EGP, which was taken as the rate measured immediately before ingestion of each meal (i.e., at 0 min for breakfast and at 240 min for lunch).

Statistics. All data are reported as means ± SE. Data were analyzed by two-way repeated-measures ANOVA with Bonferroni multiple-comparisons test used for post hoc analysis. Statistical significance was accepted when $P < 0.05$.

RESULTS

Plasma parameters. Plasma glucose excursions were statistically higher after lunch than after breakfast (Fig. 1, *A* and *B*; $P < 0.05$, main effect for meal). However, the magnitude of this effect was very small and therefore not likely of physiological significance. Although the plasma insulin responses for breakfast and lunch were similar (Fig. 1, *C* and *D*), the C-peptide responses (Fig. 1, *E* and *F*), and thus insulin secretion rates (Fig. 1, *G* and *H*), were statistically higher after lunch ($P < 0.01$, main effect for meal). Again, although these differences were statistically different, the magnitude of effect was small. Premeal glucagon levels were higher before lunch than before breakfast, but at 90 and 120 min after meal ingestion glucagon was lower after lunch compared with breakfast (Fig. 1, *I* and *J*). Active GLP-1 (7–36 amide) levels were higher before and over the initial 20 min after lunch (Fig. 1, *K* and *L*). Plasma FFA (Fig. 1, *M* and *N*) and TAG (Fig. 1, *O* and *P*) levels were higher after lunch ($P < 0.01$, main effect for diet). The pattern of FFA suppression was, however, similar after the two meals (Fig. 1*N*).

Oral vs. endogenous glucose concentrations. Enrichments for [1-^{2}H]glucose (breakfast; M+1), [U-^{13}C]glucose (lunch; M+6), and the major recycled products derived from [U-^{13}C]glucose metabolism (M+3) in the plasma are shown in Fig. 2*A*. Since glucose derived from breakfast was still circulating after lunch, total orally derived glucose concentrations (i.e., sum of oral glucose from breakfast and lunch) were slightly higher after lunch (Fig. 2*B*). Orally derived glucose excursions were statistically higher after lunch than after breakfast (Fig. 2*C*; $P < 0.01$, main effect for meal), although the magnitude of this effect was small. Endogenous glucose levels were rapidly suppressed, with the nadir (timing and magnitude) being similar for breakfast and lunch (Fig. 2*D*). However, compared with breakfast, endogenous glucose levels remained suppressed for a greater duration after lunch (Fig. 2*E*). There was little variation in ratio between [6,6-^{2}H]glucose and endogenous glucose (Fig. 2*F*), demonstrating that the variable infusion achieved a near-constant tracer-to-tracee ratio.

EGP. EGP was rapidly suppressed after both meals, with the pattern and magnitude of suppression being similar over the initial 75 min of the postmeal period (Fig. 3, *A* and *B*). However, EGP suppression after breakfast was more transient

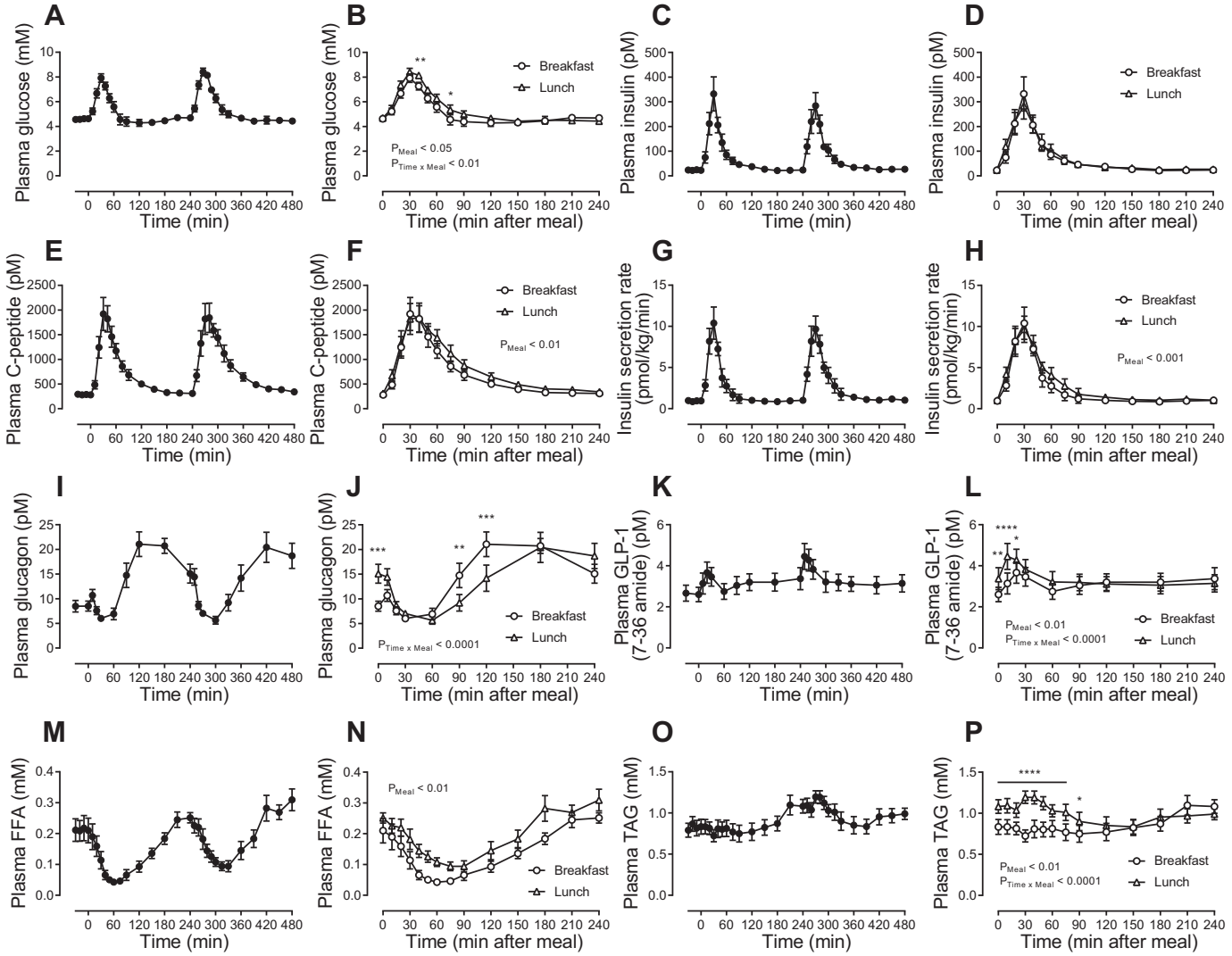

Fig. 1. Plasma metabolite and hormone concentrations after breakfast (ingested at time 0 min) and lunch (ingested at time 240 min). *A*: plasma glucose concentrations. *B*: comparison of plasma glucose responses after each meal. *C*: plasma insulin concentrations. *D*: comparison of plasma insulin responses after each meal. *E*: plasma C-peptide concentrations. *F*: comparison of plasma C-peptide responses after each meal. *G*: insulin secretion rates. *H*: comparison of insulin secretion rates after each meal. *I*: plasma glucagon concentrations. *J*: comparison of plasma glucagon responses after each meal. *K*: plasma glucagon-like peptide-1 (GLP-1; 7–36 amide) concentrations. *L*: comparison of plasma GLP-1 (7–36 amide) responses after each meal. *M*: plasma free fatty acid (FFA) concentrations. *N*: comparison of plasma FFA responses after each meal. *O*: plasma triglyceride (TAG) concentrations. *P*: comparison of plasma TAG responses after each meal. Data are means ± SE. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$.

compared with that after lunch, with EGP returning to basal at 180 min after breakfast (Fig. 3, *A* and *B*). In contrast, EGP remained suppressed for the entire postlunch period (Fig. 3, *A* and *B*) such that the percent suppression of EGP after lunch was approximately twofold greater than after breakfast (20 ± 2% vs. 39 ± 3% for breakfast vs. lunch; $P < 0.0001$). To help explain the differences in the duration of EGP suppression with breakfast vs. lunch, plasma glucagon was expressed relative to the respective premeal levels (Fig. 3*C*). Glucagon was suppressed over the initial 60 min after both meals. Consistent with the EGP response, postbreakfast glucagon levels thereafter increased and were approximately twofold higher than premeal levels for all remaining time points (Fig. 3*C*). After lunch, however, glucagon remained suppressed for longer, returning to premeal levels and not exceeding them (Fig. 3*C*).

DISCUSSION

By providing the first assessment of EGP in response to sequential mixed meals, we reveal a number of unique insights about the regulation of postprandial EGP. First, the suppression of EGP was more transient after breakfast than with lunch. After breakfast, EGP returned to basal rates within 3 h and remained at these levels until ingestion of lunch. After lunch, however, EGP remained below basal rates for the entire postmeal period. The mechanism for this observation does not appear to be related to potential effects of glucose and insulin on EGP since the excursions for glucose and insulin were similar for both meals. In contrast, the relative change in glucagon (Fig. 3*C*) was substantially different between meals and may, in part, explain the observed differences in the duration of EGP suppression. Indeed, from 90 to 180 min after

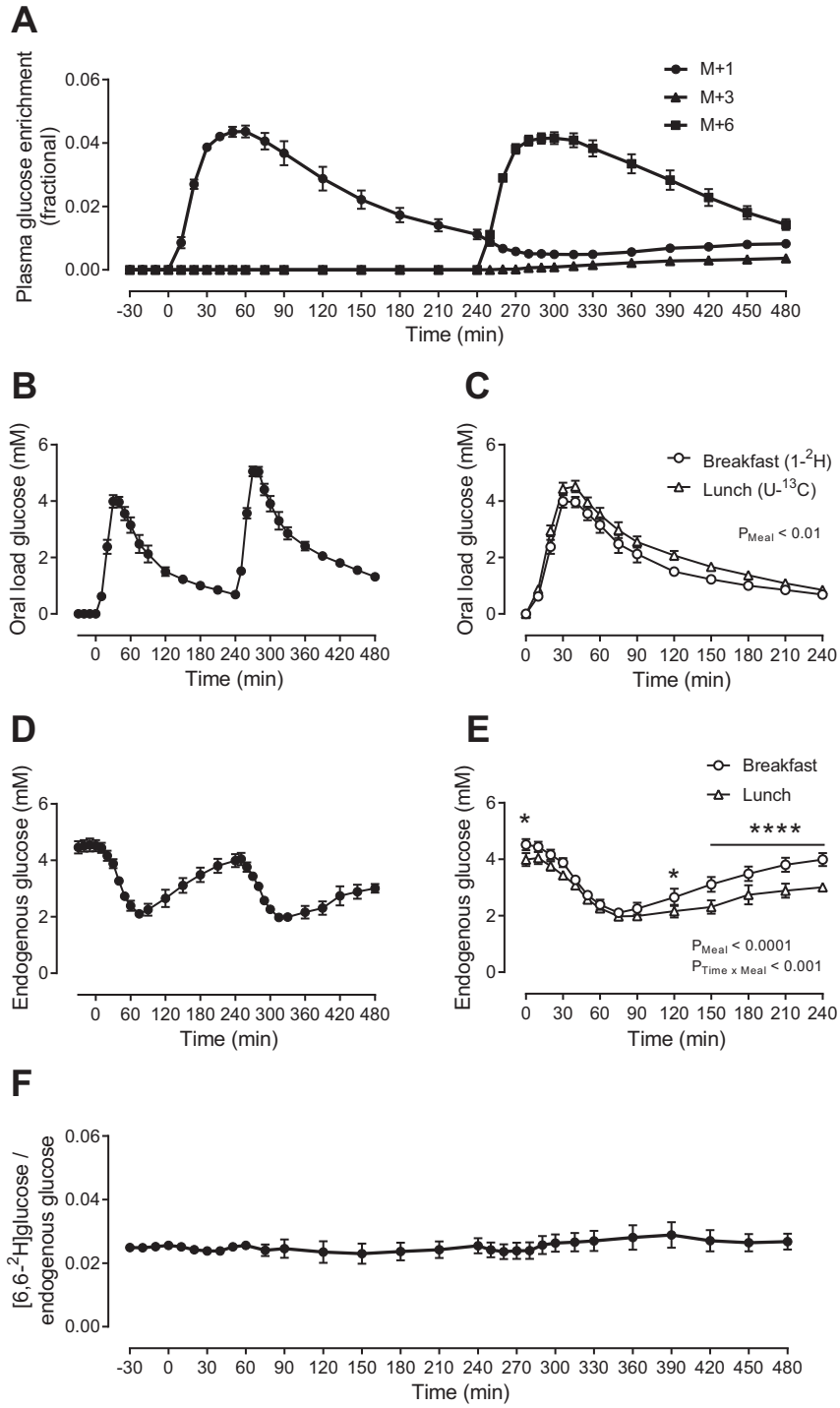

Fig. 2. Enrichment and concentrations of orally derived glucose as well as endogenous glucose concentrations and tracer-to-tracee ratios observed after breakfast (ingested at time 0 min) and lunch (ingested at time 240 min). *A*: enrichments for [1-^{2}H]glucose (breakfast; M+1), [U-^{13}C] glucose (lunch; M+6), and the major recycled products derived from [U-^{13}C]glucose metabolism (M+3) in the plasma. *B*: total oral load plasma glucose concentrations. *C*: comparison of oral load glucose concentrations after each meal. *D*: endogenous glucose concentrations. *E*: comparison of endogenous glucose concentrations after each meal. *F*: ratio between [6,6-^{2}H]glucose and endogenously derived glucose. Data are means ± SE. **P* < 0.05, *****P* < 0.0001.

breakfast, at a time when EGP was increasing toward basal, plasma glucagon was approximately twofold higher than pre-breakfast levels. In contrast, glucagon remained suppressed for a longer duration after lunch, returning to prelunch levels and not exceeding them over the final hour of the study. However, it is of interest to note that even though glucagon levels over the final hour after lunch were approximately twofold higher than fasting levels at the beginning of the day, EGP was still suppressed below basal rates at this time. Consistent with our findings, although to a smaller degree, Saad et al. (25) reported that EGP suppression was greater after lunch than after breakfast, which may have also been mediated by distinct glucagon responses to each meal. However, unlike our study, where EGP was examined in response to sequential meals on a single day, Saad et al. (25) measured glucose fluxes after breakfast and lunch on separate days.

Despite the difference in the duration of suppression, the pattern of EGP suppression was similar over the first 75 min for both meals, where a similar nadir (~50% of the basal rate) was reached. Consistent with our previous findings examining

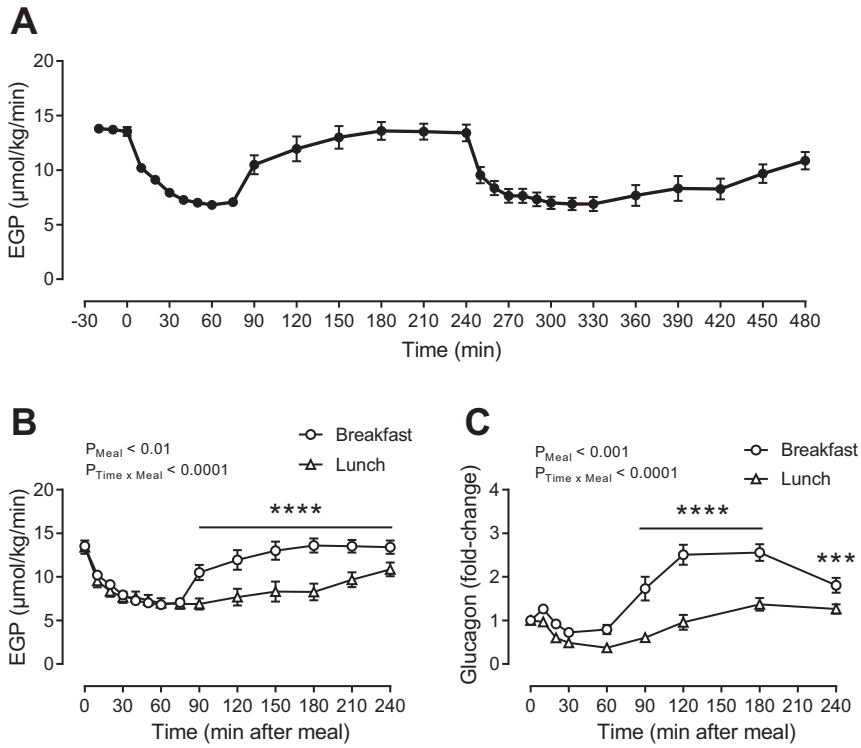

Fig. 3. Rates of endogenous glucose production (EGP) and relative change in plasma glucagon levels. *A*: EGP rates after breakfast (ingested at time 0 min) and lunch (ingested at time 240 min). *B*: comparison of EGP rates after each meal. *C*: comparison of plasma glucagon responses after each meal expressed relative to respective premeal levels. Data are means ± SE. $***P < 0.001$, $****P < 0.0001$.

the EGP dose response to ingested glucose (15), there seems to be a threshold whereby EGP cannot be further suppressed under postprandial conditions, likely because of the constitutive activity of gluconeogenesis (14, 21). Furthermore, this degree of suppression can be achieved even with meals containing modest (25–30 g) amounts of carbohydrate (15, 26). Although increasing the carbohydrate content does not appear to influence the degree or nadir of EGP suppression, the duration of suppression seems modifiable. Indeed, unlike our findings here, where EGP returned to basal 3 h after breakfast, we recently showed that despite reaching a nadir similar to that reported here (~50% of basal), EGP was suppressed for at least 4.5 h after ingestion of a mixed meal containing larger amounts (i.e., 75 g) of glucose (19). Under postprandial conditions, it therefore appears that the duration of suppression (i.e., time below basal rates), rather than the nadir, is the major modifiable component of EGP suppression (15, 19).

The findings in relation to the transient nature of postprandial EGP suppression following breakfast contrast with our previous work, where EGP remained suppressed for at least 3 h after ingestion of 25 g of pure glucose (15) despite reaching a nadir similar to that reported here. A likely explanation is the distinct plasma glucagon responses. After ingestion of 25 g of pure glucose (15), glucagon was suppressed throughout the postprandial period, only returning to basal levels at 3 h. In the present study, however, where 25 g of glucose was consumed as part of a mixed meal, glucagon was modestly suppressed over the first hour but increased above basal (~2-fold) for the remainder of the postbreakfast period. Similar responses have previously been observed when comparing the ingestion of glucose alone or as part of a mixed meal (4) and are almost certainly due to the stimulation of glucagon secretion by amino acids liberated from the protein within the mixed meal (20, 22). Thus it seems that the ingestion of other nutrients with carbohydrate can influence EGP with respect to the duration of suppression.

Interestingly, while the glycemic and insulin responses were similar for both meals, EGP was clearly different. However, although speculative, if the first meal was consumed at lunch (i.e., skipping breakfast) it is possible that EGP suppression might have been different from what was observed, revealing a potential effect of circadian rhythm and/or extended fasting duration. In addition, although we did not measure meal glucose appearance or disposal rates because of the requirement for additional tracer infusions, our findings suggest that the enhanced duration of EGP suppression with the second meal must be accompanied by lower rates of meal glucose appearance and/or disposal. If this was not the case, then glycemic excursions would be expected to be lower after lunch. Thus it appears that similar glycemic excursions can be achieved via different regulatory flux control mechanisms.

Of note, the glycemic responses following breakfast and lunch were similar. Although statistical analysis revealed that the plasma glucose excursions were in fact higher after lunch than after breakfast, the magnitude of this effect was small and is unlikely to be of physiological significance. This observation contrasts with the literature demonstrating that glycemic responses are improved after sequential meals or glucose loading (6, 7, 13, 29, 32), known as the second meal effect. The reason for this discrepancy is not apparent but may be related to the size of the glucose load or the amount of carbohydrate ingested with each mixed meal. In comparison to the relatively small amounts of carbohydrate ingested here (25 g), studies demonstrating the presence of the second meal phenomenon have typically used carbohydrate-rich (i.e., 75–200 g) test meals (6, 7, 13). In addition, it has been suggested that the glucose response to sequential meals may be affected by the fat content of the prior meal and the resultant increase in plasma TAG (10,

11). Indeed, there is evidence that after ingestion of a moderately high-fat breakfast there is a tendency for an increased glycemic response to lunch (10). Since the meals used here were almost matched on a gram basis for carbohydrate and fat, and as plasma TAG levels were higher before lunch, it is possible that the absence of the second meal effect may have been mediated by the fat content of the meal and subsequent plasma TAG response. It is also possible that the modestly higher levels of FFA before and throughout the postlunch period may contribute to this. If larger amounts of carbohydrate were ingested, then FFA might have been suppressed for longer and might not have rebounded to levels above the breakfast period.

Finally, it is important to highlight some limitations associated with our approach to estimating EGP following the second meal. It is possible that during the postlunch period some degree of [6,6-^{2}H]glucose recycling from liver glycogen could have resulted in an overestimation of plasma tracer enrichment that would underestimate the true rates of EGP. However, we believe the magnitude of any potential tracer glycogen recycling effect is likely to be small for a number of reasons. First, we employed the variable infusion tracer technique where the absolute amount of [6,6-^{2}H]glucose entering the system (see Table 2) was reduced during each postmeal period, thereby limiting the potential for [6,6-^{2}H]glucose incorporation into liver glycogen. Second, since the test meals contained small amounts of glucose, it would be assumed that the amount of liver glycogen synthesis would be less than for meals containing larger amounts of carbohydrate. Thus the capacity to incorporate [6,6-^{2}H]glucose into liver glycogen would be more restricted. Third, since loss of ^{2}H from [6,6-^{2}H]glucose occurs in gluconeogenesis (23, 35) and 30–50% of postprandial liver glycogen is synthesized by gluconeogenesis via the indirect pathway (18, 27, 30), any glycogen synthesized by this route would not be doubly labeled with ^{2}H (M+2). Taken together with the fact that we achieved relatively stable tracer-to-tracee ratios, we believe our estimates of postprandial EGP are accurate and physiologically plausible. This is supported by the fact that EGP returned to basal 3 h after breakfast and remained so for another hour until lunch was eaten. Furthermore, the magnitude and rapidity of EGP suppression were similar over the initial postprandial period (i.e., first 75 min) for both meals, and these responses are consistent with previous reports examining single-meal effects (2, 3, 5, 19, 26, 28, 30).

In summary, we show that in response to sequential meals containing modest amounts of carbohydrate, EGP is suppressed for a longer duration after ingestion of a second meal. Our findings therefore indicate the potential for distinct regulation of EGP with each meal throughout the day. This may be an important consideration when extrapolating findings in relation to the regulation of EGP from single-meal studies to free-living conditions with ad libitum daily eating patterns. Moreover, our findings suggest that EGP is likely to be in a suppressed state for much of the day, particularly in the afternoon since EGP did not return to basal levels after lunch. When a third carbohydrate-containing meal and/or drink several hours after lunch (i.e., dinner) is superimposed, when EGP is likely to already be in a suppressed state, the duration of EGP suppression could potentially extend well beyond waking hours. Such a scenario likely favors hepatic nutrient storage (anabolism) and may promote liver fat accumulation.

GRANTS

T. Ang is supported by an Australian Government Research Training Program Scholarship. G. M. Kowalski is supported by an Australian Research Council Discovery Early Career Research Award (DE180100859). C. R. Bruce is supported by an Australian Research Council Future Fellowship (FT160100017).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

T.A., G.M.K., and C.R.B. conceived and designed research; T.A., G.M.K., and C.R.B. performed experiments; T.A., G.M.K., and C.R.B. analyzed data; T.A., G.M.K., and C.R.B. interpreted results of experiments; T.A., G.M.K., and C.R.B. prepared figures; T.A., G.M.K., and C.R.B. drafted manuscript; T.A., G.M.K., and C.R.B. edited and revised manuscript; T.A., G.M.K., and C.R.B. approved final version of manuscript.

REFERENCES

1. **Antoniewicz MR, Kelleher JK, Stephanopoulos G.** Measuring deuterium enrichment of glucose hydrogen atoms by gas chromatography/mass spectrometry. *Anal Chem* 83: 3211–3216, 2011. doi:10.1021/ac200012p.
2. **Basu R, Dalla Man C, Campioni M, Basu A, Klee G, Toffolo G, Cobelli C, Rizza RA.** Effects of age and sex on postprandial glucose metabolism: differences in glucose turnover, insulin secretion, insulin action, and hepatic insulin extraction. *Diabetes* 55: 2001–2014, 2006 [Erratum in *Diabetes* 55: 2665, 2006]. doi:10.2337/db05-1692.
3. **Basu R, Di Camillo B, Toffolo G, Basu A, Shah P, Vella A, Rizza R, Cobelli C.** Use of a novel triple-tracer approach to assess postprandial glucose metabolism. *Am J Physiol Endocrinol Metab* 284: E55–E69, 2003. doi:10.1152/ajpendo.00190.2001.
4. **Bock G, Dalla Man C, Campioni M, Chittilapilly E, Basu R, Toffolo G, Cobelli C, Rizza R.** Effects of nonglucose nutrients on insulin secretion and action in people with pre-diabetes. *Diabetes* 56: 1113–1119, 2007. doi:10.2337/db06-1272.
5. **Bock G, Dalla Man C, Campioni M, Chittilapilly E, Basu R, Toffolo G, Cobelli C, Rizza R.** Pathogenesis of pre-diabetes: mechanisms of fasting and postprandial hyperglycemia in people with impaired fasting glucose and/or impaired glucose tolerance. *Diabetes* 55: 3536–3549, 2006. [Erratum in *Diabetes* 56: 549, 2007.] doi:10.2337/db06-0319.
6. **Bonuccelli S, Muscelli E, Gastaldelli A, Barsotti E, Astiarraga BD, Holst JJ, Mari A, Ferrannini E.** Improved tolerance to sequential glucose loading (Staub-Traugott effect): size and mechanisms. *Am J Physiol Endocrinol Metab* 297: E532–E537, 2009. doi:10.1152/ajpendo.00127.2009.
7. **Carey PE, Halliday J, Snaar JE, Morris PG, Taylor R.** Direct assessment of muscle glycogen storage after mixed meals in normal and type 2 diabetic subjects. *Am J Physiol Endocrinol Metab* 284: E688–E694, 2003. doi:10.1152/ajpendo.00471.2002.
8. **Cherrington AD.** Banting Lecture 1997. Control of glucose uptake and release by the liver in vivo. *Diabetes* 48: 1198–1214, 1999. doi:10.2337/diabetes.48.5.1198.
9. **Dalla Man C, Rizza RA, Cobelli C.** Meal simulation model of the glucose-insulin system. *IEEE Trans Biomed Eng* 54: 1740–1749, 2007. doi:10.1109/TBME.2007.893506.
10. **Frape DL, Williams NR, Scriven AJ, Palmer CR, O'Sullivan K, Fletcher RJ.** Diurnal trends in responses of blood plasma concentrations of glucose, insulin, and C-peptide following high- and low-fat meals and their relation to fat metabolism in healthy middle-aged volunteers. *Br J Nutr* 77: 523–535, 1997. doi:10.1079/BJN19970054.
11. **Gonzalez JT.** Paradoxical second-meal phenomenon in the acute postexercise period. *Nutrition* 30: 961–967, 2014. doi:10.1016/j.nut.2013.12.001.
12. **Hovorka R, Soons PA, Young MA.** ISEC: a program to calculate insulin secretion. *Comput Methods Programs Biomed* 50: 253–264, 1996. doi:10.1016/0169-2607(96)01755-5.
13. **Jovanovic A, Leverton E, Solanky B, Ravikumar B, Snaar JE, Morris PG, Taylor R.** The second-meal phenomenon is associated with enhanced muscle glycogen storage in humans. *Clin Sci (Lond)* 117: 119–127, 2009. doi:10.1042/CS20080542.
14. **Kowalski GM, Bruce CR.** The regulation of glucose metabolism: implications and considerations for the assessment of glucose homeostasis in

rodents. *Am J Physiol Endocrinol Metab* 307: E859–E871, 2014. doi:10.1152/ajpendo.00165.2014.

15. **Kowalski GM, Moore SM, Hamley S, Selathurai A, Bruce CR.** The effect of ingested glucose dose on the suppression of endogenous glucose production in humans. *Diabetes* 66: 2400–2406, 2017. doi:10.2337/db17-0433.
16. **Lee WN, Bassilian S, Guo Z, Schoeller D, Edmond J, Bergner EA, Byerley LO.** Measurement of fractional lipid synthesis using deuterated water (^2H_2O) and mass isotopomer analysis. *Am J Physiol Endocrinol Metab* 266: E372–E383, 1994. doi:10.1152/ajpendo.1994.266.3.E372.
17. **Livesey G, Wilson PD, Dainty JR, Brown JC, Faulks RM, Roe MA, Newman TA, Eagles J, Mellon FA, Greenwood RH.** Simultaneous time-varying systemic appearance of oral and hepatic glucose in adults monitored with stable isotopes. *Am J Physiol Endocrinol Metab* 275: E717–E728, 1998. doi:10.1152/ajpendo.1998.275.4.E717.
18. **Magnusson I, Chandramouli V, Schumann WC, Kumaran K, Wahren J, Landau BR.** Pathways of hepatic glycogen formation in humans following ingestion of a glucose load in the fed state. *Metabolism* 38: 583–585, 1989. doi:10.1016/0026-0495(89)90221-7.
19. **Morrison DJ, Kowalski GM, Grespan E, Mari A, Bruce CR, Wadley GD.** Measurement of postprandial glucose fluxes in response to acute and chronic endurance exercise in healthy humans. *Am J Physiol Endocrinol Metab* 314: E503–E511, 2018. doi:10.1152/ajpendo.00316.2017.
20. **Müller WA, Faloona GR, Aguilar-Parada E, Unger RH.** Abnormal alpha-cell function in diabetes. Response to carbohydrate and protein ingestion. *N Engl J Med* 283: 109–115, 1970. doi:10.1056/NEJM197007162830301.
21. **Nuttall FQ, Ngo A, Gannon MC.** Regulation of hepatic glucose production and the role of gluconeogenesis in humans: is the rate of gluconeogenesis constant? *Diabetes Metab Res Rev* 24: 438–458, 2008. doi:10.1002/dmrr.863.
22. **Ohneda A, Parada E, Eisentraut AM, Unger RH.** Characterization of response of circulating glucagon to intraduodenal and intravenous administration of amino acids. *J Clin Invest* 47: 2305–2322, 1968. doi:10.1172/JCI105916.
23. **Radziuk J, Pye S.** Quantitation of basal endogenous glucose production in Type II diabetes: importance of the volume of distribution. *Diabetologia* 45: 1053–1084, 2002. doi:10.1007/s00125-002-0841-6.
24. **Rizza RA, Toffolo G, Cobelli C.** Accurate measurement of postprandial glucose turnover: why is it difficult and how can it be done (relatively) simply? *Diabetes* 65: 1133–1145, 2016. doi:10.2337/db15-1166.
25. **Saad A, Dalla Man C, Nandy DK, Levine JA, Bharucha AE, Rizza RA, Basu R, Carter RE, Cobelli C, Kudva YC, Basu A.** Diurnal pattern to insulin secretion and insulin action in healthy individuals. *Diabetes* 61: 2691–2700, 2012. doi:10.2337/db11-1478.
26. **Sathananthan M, Shah M, Edens KL, Grothe KB, Piccinini F, Farrugia LP, Micheletto F, Man CD, Cobelli C, Rizza RA, Camilleri M, Vella A.** Six and 12 weeks of caloric restriction increases β cell function and lowers fasting and postprandial glucose concentrations in people with type 2 diabetes. *J Nutr* 145: 2046–2051, 2015. doi:10.3945/jn.115.210617.
27. **Shulman GI, Cline G, Schumann WC, Chandramouli V, Kumaran K, Landau BR.** Quantitative comparison of pathways of hepatic glycogen repletion in fed and fasted humans. *Am J Physiol Endocrinol Metab* 259: E335–E341, 1990. doi:10.1152/ajpendo.1990.259.3.E335.
28. **Singhal P, Caumo A, Carey PE, Cobelli C, Taylor R.** Regulation of endogenous glucose production after a mixed meal in type 2 diabetes. *Am J Physiol Endocrinol Metab* 283: E275–E283, 2002. doi:10.1152/ajpendo.00424.2001.
29. **Staub H.** Untersuchungen uber den zuckerstoffwechsel des munchen. *Z Klin Med* 91: 44–48, 1921.
30. **Taylor R, Magnusson I, Rothman DL, Cline GW, Caumo A, Cobelli C, Shulman GI.** Direct assessment of liver glycogen storage by ^{13}C nuclear magnetic resonance spectroscopy and regulation of glucose homeostasis after a mixed meal in normal subjects. *J Clin Invest* 97: 126–132, 1996. doi:10.1172/JCI118379.
31. **Toffolo G, Dalla Man C, Cobelli C, Sunehag AL.** Glucose fluxes during OGTT in adolescents assessed by a stable isotope triple tracer method. *J Pediatr Endocrinol Metab* 21: 31–45, 2008. doi:10.1515/JPEM.2008.21.1.31.
32. **Traugott K.** Uber das verhalten des blutzucker spiegels bei wiederholter unf verschiedener art enteraler zuckerzufuhr und dessen bedeutung fur die leberfunktion. *Klin Wochenschr* 1: 892–894, 1922. doi:10.1007/BF01715866.
33. **Vella A, Rizza RA.** Application of isotopic techniques using constant specific activity or enrichment to the study of carbohydrate metabolism. *Diabetes* 58: 2168–2174, 2009. doi:10.2337/db09-0318.
34. **Wolfe RR, Allsop JR, Burke JF.** Glucose metabolism in man: responses to intravenous glucose infusion. *Metabolism* 28: 210–220, 1979. doi:10.1016/0026-0495(79)90066-0.
35. **Wolfe RR, Chinkes DL.** *Isotope Tracers in Metabolic Research. Principles and Practice of Kinetic Analysis*. Hoboken, NJ: Wiley, 2005.