Postprandial aminogenic insulin and glucagon secretion can stimulate glucose flux in humans

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Abstract

Insulin and glucagon exert opposing actions on glucose metabolism and their secretion is classically viewed as being inversely regulated. This is, however, context specific as protein ingestion concomitantly stimulates euglycemic insulin and glucagon secretion. It remains enigmatic how euglycemia is preserved under these conditions. Accordingly, we examined the systems-level mechanisms governing such endocrine control of glucose homeostasis. Eight healthy participants completed a water (control) and multi-dose whey protein ingestion trial designed to augment the protein-induced endocrine response. Glucose kinetics were measured using stable isotope tracer methodology. Protein ingestion induced marked hyperaminoacidemia, hyperinsulinemia (~6-fold basal) and unprecedented hyperglucagonemia (~8-fold basal) whilst suppressing free fatty acids. Both glucose disposal (Rd) and endogenous glucose production (EGP) increased by ~25%, thereby maintaining euglycemia. This demonstrates: 1) protein ingestion can stimulate glucose Rd and EGP; 2) postprandial inhibition of adipose lipolysis does not suppress EGP; and 3) physiological hyperglucagonemia can override the hepatic actions of insulin, rendering the liver unresponsive to insulin-mediated EGP suppression. Finally, we argue that glucagon is a bona fide postprandial hormone that evolved to concurrently and synergistically work with insulin to regulate glucose, amino acid and nitrogen metabolism. These findings may have implications for glucagon receptor antagonist or agonist-based therapies.

Insulin and glucagon are master regulators of glucose homeostasis [1]. Dysfunction in the secretion and action of both hormones drives hyperglycemia in type 1 and 2 diabetes [1]. Therefore, understanding how insulin and glucagon interact and are integrated at the systems level is of fundamental significance. Classically, insulin and glucagon are described as exerting opposing actions on their target tissue(s), while their secretion is also considered to be inversely regulated [1]. Hence, any increase in insulin secretion is normally accompanied by glucagon suppression, thereby ensuring a tightly regulated feedback loop that maintains glucose homeostasis [1].

While the reciprocal regulation of insulin and glucagon secretion is apparent after carbohydrate ingestion [1], there are in fact circumstances when this relationship does not hold. Indeed, dietary protein ingestion alone concurrently stimulates both insulin and glucagon secretion [2, 3]. Under these conditions, insulin and glucagon simultaneously exert agonistic and antagonistic actions on metabolic homeostasis. In relation to amino acid metabolism, through its inhibitory actions on muscle proteolysis, insulin stimulates muscle amino acid uptake [4], while glucagon stimulates hepatic amino acid uptake, catabolism and ureagenesis [5]. Thus, through independent actions on the muscle (insulin) and liver (glucagon), these hormones synergistically promote plasma amino acid clearance and postprandial amino acid disposal. However, insulin and glucagon exert opposing actions on hepatic glucose metabolism [1], while insulin also promotes tissue glucose uptake. Notably, despite this complex endocrine interaction, and the fact that amino acids are substrates for gluconeogenesis, protein ingestion does not affect blood glucose levels [3]. While the maintenance of euglycemia following protein ingestion is a well-known phenomenon [2, 3], the mechanisms involved remain enigmatic.

Since insulin suppresses endogenous glucose production (EGP) and stimulates glucose disposal (Rd), protein-induced hyperinsulinemia would be expected to lower blood glucose;

yet this does not occur. It therefore seems that protein-induced hyperglucagonemia antagonizes the glucose-lowering actions of insulin to prevent hypoglycemia [6]. Interestingly, in both healthy people and those with type 2 diabetes, glucose fluxes (EGP and Rd) remain unaltered following protein ingestion [7-9]. Conversely, in individuals with type 1 diabetes, the lack of endogenous insulin secretion yet persistence of protein-induced glucagon secretion stimulates EGP, causing blood glucose to increase [7]. Thus, in people with an intact endocrine pancreas, it appears that protein-induced postprandial hyperglucagonemia precisely counteracts insulin's suppressive actions on EGP while Rd is not stimulated, thereby maintaining euglycemia.

While the abovementioned studies in healthy individuals [7-9] failed to demonstrate changes in glucose flux following protein ingestion, this does not preclude the potential for such effects to occur. It is possible that the magnitude and duration of the hormonal response following protein ingestion (meat or eggs) in the previous studies [7-9] was insufficient to surpass the threshold to alter glucose flux. Evidently, not all dietary protein sources are equipotent at stimulating insulin and glucagon secretion, with the speed of digestion and rate of systemic amino acid entry being key determinants of the hormonal response [10]. For example, slow-absorbing egg proteins exert negligible responses [9, 11], meat proteins exert modest responses [2, 8] while dairy proteins [11], particularly the whey fraction [12-14], robustly and rapidly stimulate insulin and glucagon secretion. With this in mind, we devised a physiologic paradigm using fast-absorbing whey protein to examine whether the concurrent induction of sustained hyperinsulinemia and hyperglucagonemia has the capacity to alter glucose fluxes in healthy humans during the postprandial euglycemic state. From a glucoregulatory perspective, this may reveal unique insight into the metabolic 'tug-of-war' between insulin and glucagon action. This is particularly relevant since glucagon receptor antagonism and ironically agonism (co-agonist therapy) are emerging as therapeutic strategies for diabetes and obesity.

RESEARCH DESIGN AND METHODS

Participants

Eight healthy individuals (4 females/4 males; 30 ± 2 years; 66 ± 4 kg; 22 ± 1 kg/m^2) participated in this study which was approved by the Deakin University Human Research Ethics Committee. The purpose, nature, and potential risks were explained, and informed consent obtained.

Study Design

Participants completed two trials in random order, separated by 1-2 weeks, where whey protein or water was ingested. A standardized diet (8,465 kJ; 52% carbohydrate, 18% protein and 30% fat) was consumed on the day before each trial. Trials commenced following a 10-12 h overnight fast, consuming only water from 2130 h the evening prior. Strenuous exercise was avoided for 48 h prior. Upon arrival at the laboratory at 0700 h, height and weight were recorded. A 22-gauge cannula was inserted into a dorsal hand vein in a retrograde fashion for arterialized blood sampling using a heated box (50-55°C). A second cannula was placed in the contralateral forearm vein for [6,6-^{2}H]glucose infusion (Cambridge Isotope Laboratories, Inc., Tewksbury, MA). A primed (33 μmol/kg infused over 5 min) continuous infusion of [6,6-^{2}H]glucose (0.33 μmol/kg/min) commenced at 0730 h. After the prime, [6,6-^{2}H]glucose was infused at a constant rate for 120 min (-120 to 0 min) and continued throughout the experimental period (0 to 240 min).

At 0930 h (0 min), participants commenced either the protein-feeding or water control trials. During the protein trial, 25 g of whey protein isolate (True Protein, Brookvale, NSW, Australia; French vanilla flavour; <1 g carbohydrate per serve) dissolved in 200 ml of water was ingested at 0, 30 and 60 min (total 75 g protein ingested). During the control trial, participants ingested 200 ml of water at 0, 30 and 60 min. Arterialized blood was collected in EDTA-containing

vacutainers at 7.5, 15, 22.5, 30, 37.5, 45, 52.5, 60, 67.5, 75, 82.5, 90, 105, 120, 135, 150, 180, 210 and 240 min. For glucagon, samples were collected in BD™ P800 vacutainers.

Plasma Analysis

Plasma glucose, glycerol, amino acids, urea and β-hydroxybutyrate were measured via GC-MS, with absolute concentrations calculated from linear regression of serially diluted external unlabeled standards using the isotope dilution technique whereby all samples were spiked with stable isotope labeled internal standards (see additional online material for more detail). Plasma [6,6-^{2}H]glucose enrichment and total glucose concentration was determined using the glucose methyloxime pentaproprionate derivative via positive chemical ionization GC-MS with raw data corrected for natural isotopic background abundance skew (see additional online material for more detail).

Commercially available ELISA kits were used to determine plasma insulin (ALPCO, Salem, NH), glucagon (Mercodia, Uppsala, Sweden) and C-peptide (Millipore, Billerica, MA). Plasma free fatty acids (FFA; Wako Chemicals, Richmond, VA) and triglycerides (Roche Diagnostics, Basel, Switzerland) were determined via spectrophotometric assay.

Calculations and Statistics

Insulin secretion rates (ISR) were calculated by C-peptide deconvolution and glucose fluxes were modelled using Steele's non-steady state equations (see additional online material for more detail). All data are reported as mean±SEM. Two-way repeated measures ANOVA and paired sample t-tests were used where appropriate. Bonferroni multiple comparisons test was used for post-hoc analysis. Significance was accepted when $P<0.05$.

RESULTS

Protein ingestion had no effect on plasma glucose (Fig 1A), yet there was a rapid and sustained insulin response (Fig 1B), with C-peptide and insulin secretion rates following a similar pattern (Fig 1C & D). Protein ingestion also caused marked hyperglucagonemia (Fig 1E). While the kinetics and magnitude of the insulin and glucagon responses were similar (~6-fold basal) over the first 60 min after protein ingestion (Fig 1F), insulin gradually declined thereafter whereas glucagon continued to rise, peaking at ~8-fold basal at 120 min (Fig 1F). In relative terms, the glucagon response was larger than that of insulin, while the C-peptide response was substantially smaller than both insulin and glucagon (Fig 1F). While plasma triglycerides were unaffected by protein ingestion (Fig 1G), FFAs and glycerol (Fig 1H & I) were markedly suppressed.

Plasma essential (Fig 2A-H) and non-essential (Fig 3A-J) amino acids rapidly increased following protein ingestion with the branched-chain amino acids, particularly leucine (~6-fold basal) and isoleucine (~9-fold basal) showing the greatest response (Fig 2A & B). Additionally, plasma urea progressively increased following protein ingestion (Fig 3K). β-hydroxybutyrate levels increased gradually during both the water and protein trials, with no significant differences between either condition (Fig 3L). Following protein ingestion there was a progressive increase in glucose flux, with EGP and Rd both increasing by ~25% (Fig 4A-C). As a result, on average ~11 g of additional glucose entered the circulation during the 4-h protein ingestion trial (Fig 4D).

DISCUSSION

Previous single-dose whey protein ingestion studies have shown robust, yet transient euglycemic insulin and glucagon responses [12-14]. Here, we employed a multi-dose protein feeding strategy so as to potentiate the duration of this endocrine response to maximize the opportunity for insulin and glucagon to alter metabolism. As intended, this feeding paradigm caused a rapid and sustained euglycemic insulin response. Interestingly, the postprandial hyperinsulinemia was mediated by increased insulin secretion and reduced insulin clearance, as evident by the lower relative C-peptide response to that of insulin. While a rise in glucagon was expected, the magnitude was unprecedented (~8-fold basal). To the best of our knowledge, this is the largest physiological, not pathological (e.g. glucagonoma) increase in glucagon that we have observed in the literature. While these hormonal responses did not impact glycemia, plasma FFAs and glycerol were suppressed, consistent with adipose tissue being highly insulin sensitive [15]. This supports the view that insulin's anti-lipolytic actions on adipose are dominant, and within physiological concentrations, glucagon has little, if any lipolytic effect [16].

As expected, protein ingestion had no effect on plasma glucose despite causing marked hyperinsulinemia and hyperglucagonemia. So how did blood glucose remain constant? Protein feeding proportionally increased both Rd and EGP, with glucose turnover increasing by ~25%, which explains the euglycemia. While the source of the extra EGP is unknown, we speculate the combined hyperaminoacidemia and hyperglucagonemia stimulated gluconeogenic flux, thus providing an energy costly pathway for hepatic and perhaps kidney amino acid catabolism [5]. To our knowledge, this is the first time pure protein ingestion has been shown to robustly stimulate Rd in healthy humans. In light of the physiologic euglycemic-hyperinsulinemic-hyperglucagonemic state (i.e. absence of hyperglycemia) [17], the additional glucose produced

was not likely taken up by the liver, but rather muscle (skeletal and cardiac) and/or adipose tissue.

Previous studies have shown that whey protein ingestion during an insulin-clamp can modestly impair insulin-stimulated glucose uptake [18]. It is therefore possible that the hyperaminoacidemia in our study prevented even greater increase in insulin-stimulated Rd from occurring. However, we do not believe this is pathological insulin resistance, but rather a physiological compensation to acutely altered substrate availability. Regardless, our feeding protocol induced a sufficient degree of hyperinsulinemia to stimulate peripheral tissue glucose uptake, even in the face of marked hyperglucagonemia. This supports the concept that physiological concentrations of glucagon do not directly oppose skeletal or cardiac muscle glucose metabolism as these tissues lack glucagon receptors [19]. Since previous studies have failed to stimulate EGP in humans in the presence of selectively high glucagon and gluconeogenic substrate concentrations, an effect attributed to hepatic autoregulation [20], we believe the increase in EGP here is secondary to insulin-stimulated Rd. We therefore propose that the increase in Rd induced a proportional increase in EGP to maintain euglycemia in the absence of exogenous glucose. Accordingly, protein-induced stimulation of glucose flux appears to be dependent on the magnitude and duration of the hyperinsulinemia, likely explaining why previous protein-feeding studies that reported modest insulin responses did not observe changes in glucose flux [7-9]. This is consistent with the notion that stimulation of peripheral (muscle) Rd is a naturally insulin resistant process requiring substantial increments in systemic insulin [15]. In regards to the multi-dose whey protein approach employed here, it is important to note that it is not representative of typical daily protein intake derived from whole food over multiple meals, but nevertheless elicits a physiological postprandial response that was intended to push the metabolic and endocrine systems in order to reveal fundamental control mechanisms that govern metabolic homeostasis and provide teleological insight.

It is interesting to comment on the direct (hepatocyte) vs. indirect (extrahepatic) effects of insulin on EGP. It has been suggested that insulin trumps glucagon at any concentration to inhibit EGP [21], and that suppression of FFAs through insulin's anti-lipolytic effects are key determinants of this response; 'single gateway hypothesis' [22]. Our data does not support this. Here, EGP increased despite marked FFA suppression, thereby dissociating control of postprandial EGP from adipose lipolysis. Furthermore, we show that postprandial hyperglucagonemia can completely override the effects of hyperinsulinemia on EGP, suggesting that the 'tug-of-war' on hepatic glucose metabolism between insulin and glucagon is hepatocyte specific (direct).

Finally, how are glucose fluxes precisely coordinated to maintain euglycemia under these conditions and should the glucagon response be viewed as glucose counter-regulatory? These are not trivial issues to decipher, but perhaps are most appropriately discussed from an evolutionary perspective. Through evolution of the endocrine system, complex life forms have integrated metabolic control across multiple effector organs (i.e. liver, kidneys, adipose, and skeletal muscle). This multi-organ control has resulted in 'hard-wired' metabolic flexibility, permitting survival across widely varying environmental conditions such as famine, feasting, and seasonal changes to macronutrient availability. Perhaps our view of insulin and glucagon biology has been heavily shaped by the context of our modern mixed macronutrient, yet dominantly high carbohydrate diets. Through multiple daily meals, dietary glucose from processed sugars and starch results in frequent glucose spikes, requiring a dominant insulin and weakened glucagon response, leading to inhibition of EGP and stimulation of Rd. As glycemia returns towards baseline, the body must ensure glucose fluxes are not overcommitted toward glucose reduction, thus requiring counter-regulatory glucagon secretion and hence appropriate EGP recovery to prevent hypoglycemia.

However, during the Paleolithic period in which humans evolved (~2.6 million-10,000 years ago) [23], many, perhaps even the majority of our hunter-gatherer ancestors would have infrequently or possibly never experienced substantial blood glucose spikes. Whether the diet was periodically or persistently absent in carbohydrate such as in cold-climate hunting populations (e.g. Inuit), or whether the diet contained modest quantities of unprocessed high fiber plant-derived food, the diets would have been by large classed as 'low glycemic' and relatively high in protein [23]. Accordingly, concurrent euglycemic protein-induced insulin and glucagon secretion may have been the 'prototypical' postprandial response, as would be expected to occur in carnivores [24] where the hyperaminoacidemia following meat-based meals likely results in substantial insulin and glucagon secretion. The insulin response would concomitantly inhibit adipose lipolysis and muscle proteolysis, while stimulating muscle amino acid and glucose uptake. Ultimately, this would favor adipose tissue lipid deposition and muscle protein and glycogen synthesis. Synchronously, given the predominant distribution of glucagon receptors at the liver and kidney [19], the combined postprandial glucagon response and hyperaminoacidemia would stimulate hepatic amino acid uptake, deamination, oxidation, gluconeogenesis and ureagenesis while enhancing urea excretion at the kidney [25]. This would stimulate plasma amino acid disposal, ammonia detoxification and hepatic glycogen synthesis likely via the indirect (gluconeogenic) pathway whilst supporting elevated EGP to maintain euglycemia. Given the absolute requirement of protein, but not carbohydrate, for mammalian survival, it could be envisioned that such endocrine and metabolic responses were evolutionarily selected for in humans. Accordingly, we believe that glucagon is a *bona fide* postprandial hormone which evolved to synergistically support insulin. Consequently, attempts to therapeutically inhibit or activate the glucagon-receptor need to carefully consider not only glucose, but also amino acid and nitrogen homeostasis.

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Data and Resource Availability

Any raw datasets generated during the current study are available from the corresponding author on reasonable request, with all reagent and analytical detail available on the online supplementary file.

Author contributions

T.A., G.M.K., and C.R.B. contributed to all aspects of the study including conceptualization and study design, data collection and analysis, and manuscript preparation. G.M.K. and C.R.B. 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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Figure legends

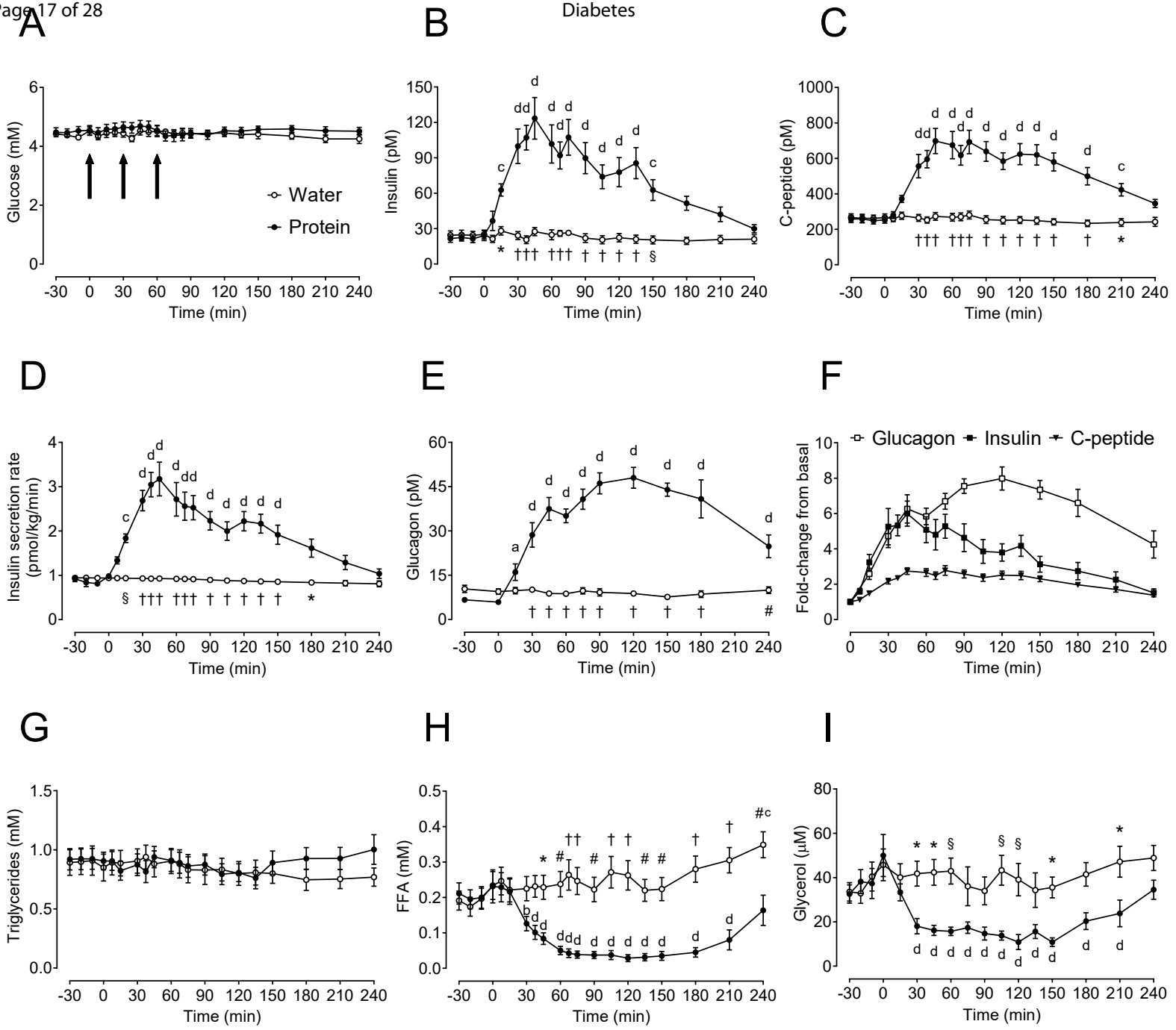
Figure 1. Plasma metabolite and hormone concentrations. Water or whey protein isolate (25 g) solutions were ingested at 0, 30 and 60 min (⬆). **A:** Glucose. **B:** Insulin ($P_{Time}<0.0001$, $P_{Treatment}<0.0001$, $P_{Interaction}<0.0001$). **C:** C-peptide ($P_{Time}<0.0001$, $P_{Treatment}<0.0001$, $P_{Interaction}<0.0001$). **D:** Insulin secretion rates ($P_{Time}<0.0001$, $P_{Treatment}<0.0001$, $P_{Interaction}<0.0001$). **E:** Glucagon ($P_{Time}<0.0001$, $P_{Treatment}<0.0001$, $P_{Interaction}<0.0001$). **F:** Fold-change kinetics relative to basal (average of -30 to 0 min time points) for insulin, glucagon and C-peptide following protein ingestion. **G:** Triglycerides ($P_{Time}<0.0001$). **H:** FFAs ($P_{Time}<0.0001$, $P_{Treatment}<0.01$, $P_{Interaction}<0.0001$). **I:** Glycerol ($P_{Time}<0.0001$, $P_{Treatment}<0.05$, $P_{Interaction}<0.0001$). Data are mean±SEM and were analysed using two-way repeated measures ANOVA followed by Bonferroni post-hoc test. Water vs protein ingestion: $^{*}P<0.05$; $^{\S}P<0.01$; $^{\#}P<0.001$; $^{\dagger}P<0.0001$. Comparisons against baseline (0 min) within trial: $^{a}P<0.05$; $^{b}P<0.01$; $^{c}P<0.001$; $^{d}P<0.0001$.

Figure 2. Plasma essential amino acid concentrations. **A:** Leucine ($P_{Time}<0.0001$, $P_{Treatment}<0.0001$, $P_{Interaction}<0.0001$). **B:** Isoleucine ($P_{Time}<0.0001$, $P_{Treatment}<0.0001$, $P_{Interaction}<0.0001$). **C:** Valine ($P_{Time}<0.0001$, $P_{Treatment}<0.0001$, $P_{Interaction}<0.0001$). **D:** Lysine ($P_{Time}<0.0001$, $P_{Treatment}<0.0001$, $P_{Interaction}<0.0001$). **E:** Methionine ($P_{Time}<0.0001$, $P_{Treatment}<0.0001$, $P_{Interaction}<0.0001$) **F:** Threonine ($P_{Time}<0.0001$, $P_{Treatment}<0.0001$, $P_{Interaction}<0.0001$). **G:** Phenylalanine ($P_{Time}<0.0001$, $P_{Treatment}<0.0001$, $P_{Interaction}<0.0001$). **H:** Histidine ($P_{Time}< 0.0001$, $P_{Treatment}< 0.001$, $P_{Interaction}<0.0001$). Data are mean±SEM and were analysed using two-way repeated measures ANOVA followed by Bonferroni post-hoc test. Water vs protein ingestion: $^{*}P<0.05$; $^{\S}P<0.01$; $^{\#}P<0.001$; $^{\dagger}P<0.0001$. Comparisons against baseline (0 min) within trial: $^{a}P<0.05$; $^{b}P<0.01$; $^{c}P<0.001$; $^{d}P<0.0001$.

Figure 3. Plasma non-essential amino acid and urea concentrations. **A:** Alanine ($P_{Time}<0.0001$, $P_{Treatment}<0.01$, $P_{Interaction}<0.0001$). **B:** Serine ($P_{Time}<0.0001$, $P_{Treatment}<0.001$, $P_{Interaction}<0.0001$). **C:** Arginine ($P_{Time}<0.0001$, $P_{Treatment}<0.05$, $P_{Interaction}<0.0001$). **D:** Aspartate ($P_{Time}<0.0001$, $P_{Treatment}<0.0001$, $P_{Interaction}<0.0001$). **E:** Glutamate ($P_{Time}<0.0001$, $P_{Treatment}<0.0001$, $P_{Interaction}<0.0001$). **F:** Glutamine ($P_{Time}<0.0001$, $P_{Treatment}<0.001$, $P_{Interaction}<0.0001$). **G:** Tyrosine ($P_{Time}<0.0001$, $P_{Treatment}<0.0001$, $P_{Interaction}<0.0001$). **H:** Proline ($P_{Time}<0.0001$, $P_{Treatment}<0.0001$, $P_{Interaction}<0.0001$). **I:** Cystine ($P_{Time}<0.0001$, $P_{Treatment}<0.0001$, $P_{Interaction}<0.0001$). **J:** Glycine ($P_{Time}<0.0001$, $P_{Interaction}<0.0001$). **K:** Urea ($P_{Time}<0.0001$, $P_{Treatment}<0.01$, $P_{Interaction}<0.0001$). **L:** β-Hydroxybutyrate ($P_{Time}<0.0001$). Data are mean±SEM and were analysed using two-way repeated measures ANOVA followed by Bonferroni post-hoc test. Water vs protein ingestion: $^{*}P<0.05$; $^{\S}P<0.01$; $^{\#}P<0.001$; $^{\dagger}P<0.0001$. Comparisons against baseline (0 min) within trial: $^{a}P<0.05$; $^{b}P<0.01$; $^{c}P<0.001$; $^{d}P<0.0001$.

Figure 4. Plasma tracer enrichment and glucose fluxes. **A:** Plasma [6,6-^{2}H]glucose enrichment ($P_{Time}<0.0001$, $P_{Treatment}<0.01$, $P_{Interaction}<0.0001$). **B:** EGP ($P_{Time}<0.0001$, $P_{Treatment}<0.01$, $P_{Interaction}<0.0001$). **C:** Rd ($P_{Time}<0.0001$, $P_{Treatment}<0.01$, $P_{Interaction}<0.0001$). **D:** Total glucose produced (paired t-test). Data are mean±SEM and were analysed using two-way repeated measures ANOVA followed by Bonferroni post-hoc test. Water vs protein ingestion: $^{*}P<0.05$;

$^{\S}P<0.01$; $^{\#}P<0.001$; $^{\dagger}P<0.0001$. Comparisons against baseline (0 min) within trial: $^{a}P<0.05$; $^{b}P<0.01$; $^{c}P<0.001$; $^{d}P<0.0001$.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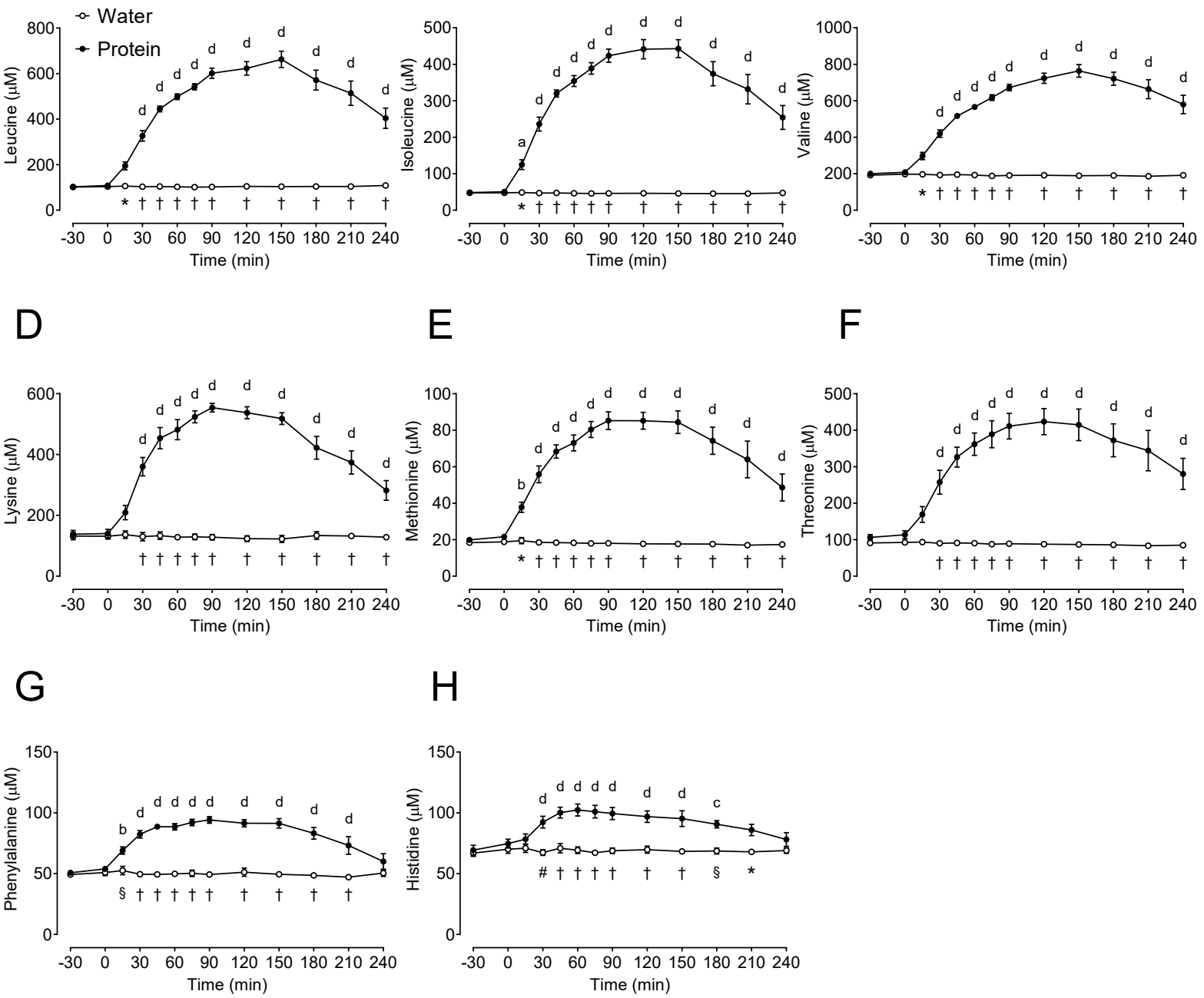
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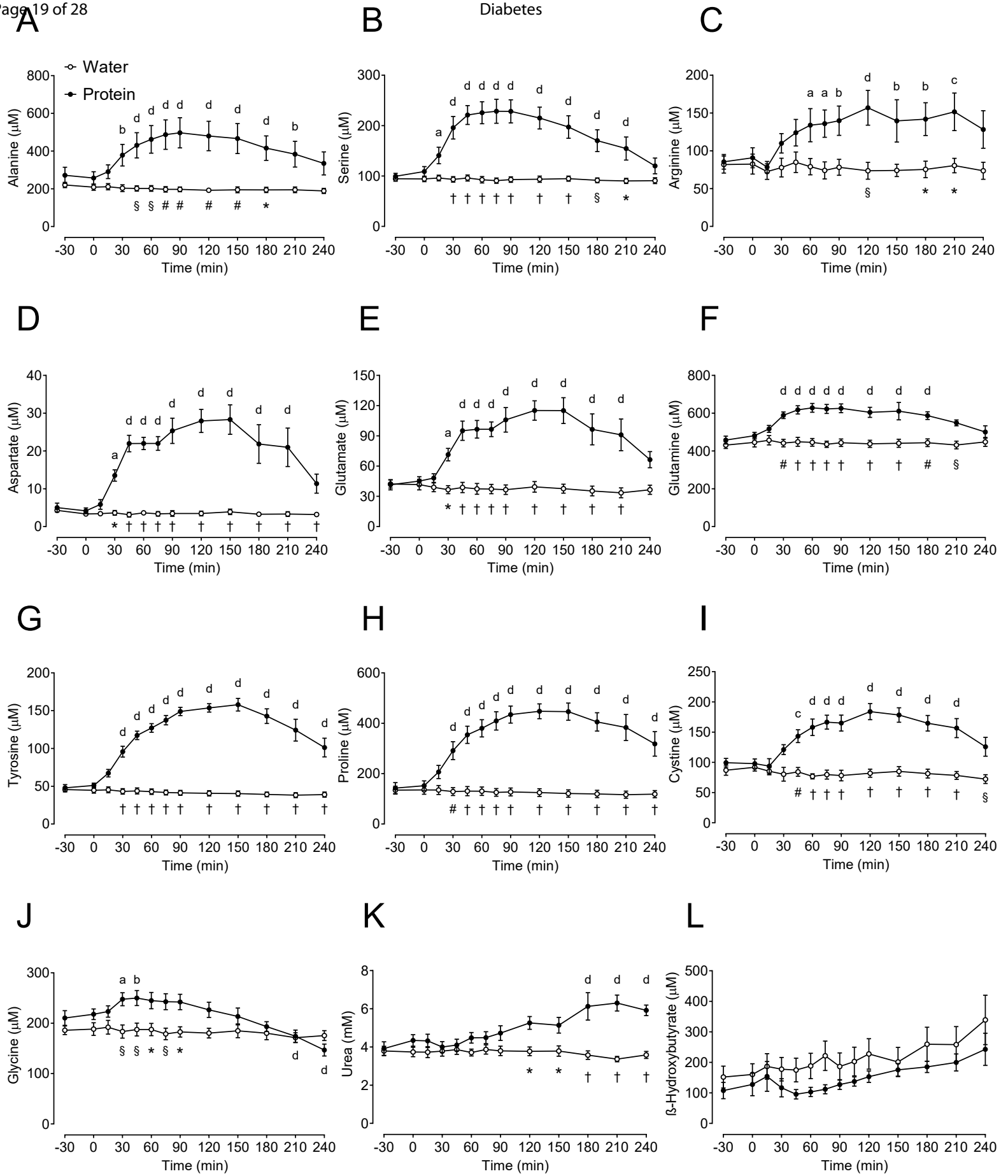
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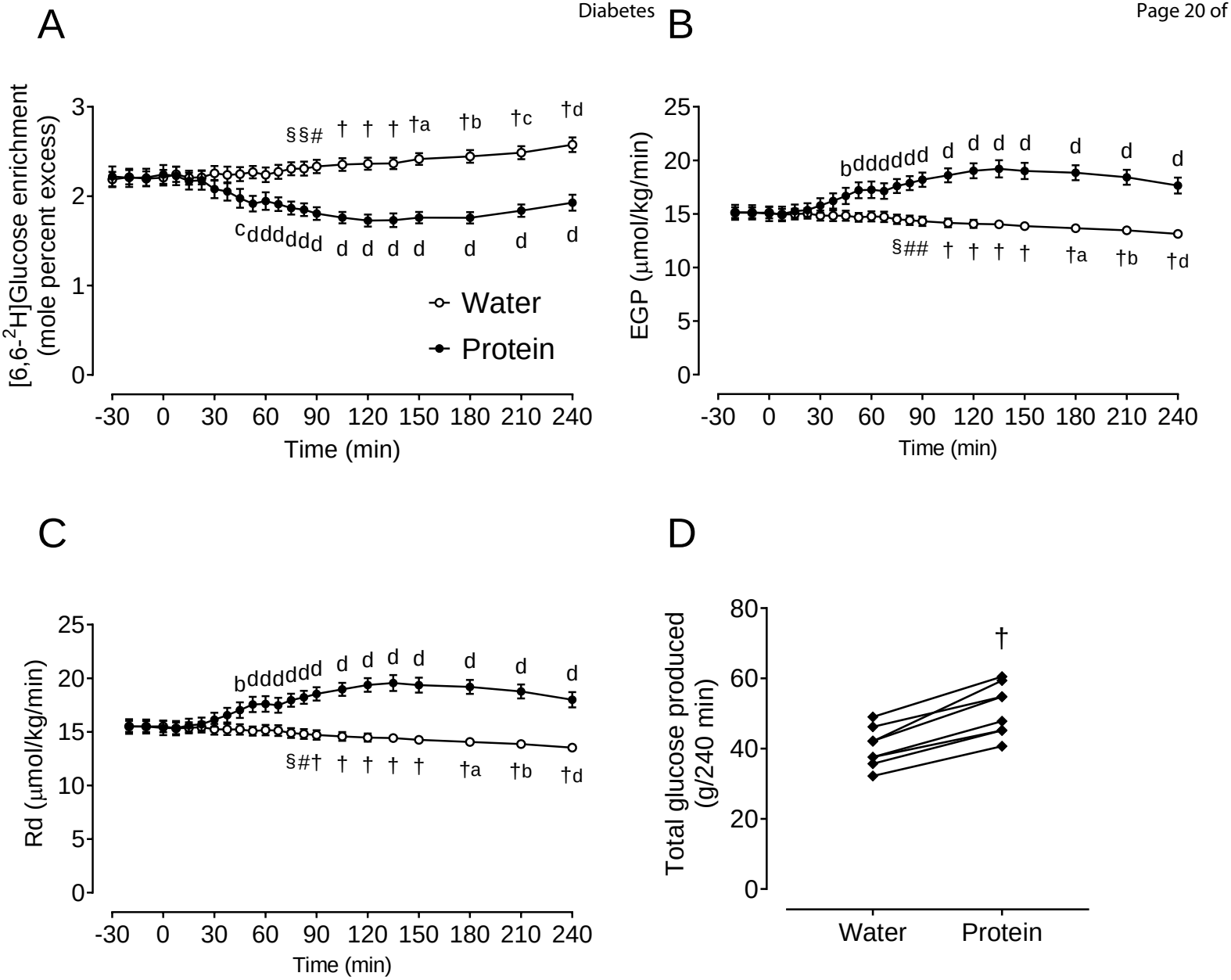
β-Hydroxybutyrate (μM)
Time (min)


A
[6,6-2H]Glucose enrichment (mole percent excess)
Time (min)
Water
Protein
B
EGP (μmol/kg/min)
Time (min)
C
Rd (μmol/kg/min)
Time (min)
D
Total glucose produced (g/240 min)
Water
Protein

Additional Material

Nutrient composition

The energy content as well as the macronutrient profile and amino acid composition of the whey protein isolate (True Protein; French Vanilla, Brookvale, NSW, Australia) used is shown in Table 1 below.

Table 1. Nutrient composition of the whey protein isolate (French Vanilla)

Nutrition composition	**Per 100 g**
Energy (kJ)	1525
Protein (g)	86.9
Carbohydrate (g)	3.3
Fat (g)	0.3
Essential amino acids (g)	
- Leucine	11.3
- Isoleucine	7.2
- Valine	6.4
- Lysine	10.3
- Methionine	2.4
- Threonine	7.4
- Phenylalanine	3.3
- Histidine	1.9
- Tryptophan	1.9
Non-essential amino acids (g)	
- Alanine	5.6
- Serine	5.1
- Arginine	2.4
- Aspartic acid	11.3
- Glutamic acid	19.0
- Tyrosine	3.4
- Proline	6.4
- Cysteine	2.9
- Glycine	1.9

Insulin secretion

Insulin secretion rates were calculated by C-peptide deconvolution using Insulin SECretion (ISEC) software [1].

Plasma glucose analysis

Plasma glucose concentrations and [6,6-^{2}H]glucose enrichment were determined via GC-MS using the glucose methyloxime pentaproprionate derivative [2, 3]. Briefly, 10 μL of plasma, serially diluted unlabelled glucose standards (0-10 mM) and extraction blanks (water) were mixed with 100 μL of methanol containing 0.2 mM [U-^{13}C]glucose (Cambridge Isotope Laboratories, Inc., Tewksbury, MA) which was used as an internal standard. Samples were vortexed and centrifuged at 15,000 rpm for 10 min at 4°C after which 80 μL of the supernatant was transferred into 250 μL glass GC inserts (Agilent Technologies, Santa Clara, CA, USA) and dried in a speed vacuum at 37°C (Labconco, MO, USA). Samples were derivatized by adding 50 μL of methoxyamine hydrochloride (Sigma-Aldrich, St. Louis, MO, USA) in pyridine (20 mg/mL; Sigma-Aldrich) followed by incubation at 90°C for 60 min. The second derivatization step was performed by adding 100 μL of propionic anhydride (Sigma-Aldrich) followed by incubation at 60°C for 30 min. Excess reagent was evaporated in a speed vacuum at 37°C and the dry residue resuspended in 100 μL of ethyl acetate for GC-MS analysis using an Agilent 7890B Gas Chromatography system attached to a VF-5ms capillary column with a 10-m inert EZ-guard (30 m, 0.25 mm, 0.25 μm, J & W Scientific) and a 5977B Mass Selective Detector (Agilent Technologies) via positive chemical ionization using methane as the reagent gas and helium as the carrier. The glucose methyloxime pentapropionate derivative was analysed by monitoring the molecular ions of 416-422 m/z, corresponding to the M0 (naturally occurring glucose; 416 m/z), M+2 ([6,6-^{2}H]glucose; 418 m/z) and M+6 ([U-^{13}C]glucose internal standard; 422 m/z) isotopomers respectively. Selective ion monitoring (SIM) was performed with a 10 ms dwell time for each ion. Samples (0.5 μL) were injected using a 20:1 split ratio. The ion abundances were determined using the Quantitative Mass Hunter Workstation (Agilent Technologies). The raw isotopomer data were corrected (mole percent excess) for natural isotopic background abundance skew using the matrix method [4].

Following correction for isotopic background abundance, absolute plasma glucose concentrations were calculated from linear regression of the serially diluted external (unlabeled) standards using the isotope dilution technique. The total glucose concentration included both the presence of unlabeled natural and infused isotope labeled glucose (i.e. sum of M0-M+2).

Plasma amino acid and urea analyses

Plasma urea and amino acid concentrations were measured via GC-MS. Specifically, 100 μL of plasma, serially diluted unlabeled external standards containing urea (0-10 mM; Bio-Rad Laboratories Pty. Ltd., Gladesville, NSW, AU; 1610730), primary amino acid mix (0-1 mM; Cambridge Isotope Laboratories; MSK-A2-US-1.2) and L-glutamine (0-1 mM; Sigma-Aldrich; G8540-25G) and extraction blanks (water) were spiked with 5 μL of a stable isotope labeled internal standard mix containing 1.25 mM uniformly ^{13}C and ^{15}N labelled urea (Cambridge Isotope Laboratories, Inc.; CNLM-234-PK), 1.25 mM primary amino acid mix (Cambridge Isotope Laboratories, Inc.; MSK-A2-1.2) and 1.25 mM $^{13}C_5$ glutamine (Cambridge Isotope Laboratories, Inc.; CLM-1822-H-0.5), after which all samples were vortexed and placed on ice. Working in batches of six samples at a time, 100 μL of 20 mM HCl in 10% N-propanol (reagent one) was added to each sample to lower the pH for subsequent clean-up via a modified solid-phase cation-exchange chromatography procedure using EZ:Faast™ Amino Acid Analysis sorbent tips (Phenomenex, Lane Cove, NSW, Australia). The pH-adjusted samples were passed through the resin using 1.5 mL syringes. After discarding reagent one, the resin-filled tips were washed by passing 200 μL of 30% N-propanol solution (reagent two) as with the previous step. The resin-bound amino acids were eluted from the tip by drawing up 200 μL of 2N NH_4OH and expelling the resin into 1.5 mL Eppendorf tubes using 0.6 mL syringes. Samples were thoroughly vortexed, centrifuged at 15,000 rpm for 5 min at 4°C and

then 150 µL of resin-free NH_4OH supernatant transferred into 250 µL glass GC inserts. After complete evaporation at 37°C in a speed vacuum, the dry residue was derivatized by adding 25 µL of pyridine (Sigma-Aldrich) and 25 µL of N-methyl-N-(tert-butyldimethylsilyl)trifluoroacetamide with 1% tertbutyldimethylchlorosilane (Sigma-Aldrich), followed by incubation at 60°C for 30 min. The tert-butyldimethylsilyl (TBDMS) derivatized samples were injected (2 µL splitless; 50 mL/min purge flow; 0.5 min purge time) into an Agilent 6890N GC system connected to a VF-5ms capillary column with a 10-m inert EZ-guard (30 m, 0.25 mm, 0.25 µm) and Agilent 5975C MSD (Agilent Technologies). Samples were analysed in the electron ionization (EI) mode, with helium as the carrier gas. The GC front inlet was set to 250°C and transfer line to 270°C, while the MS quadrupole and source temperatures were set to 150°C and 230°C, respectively. The oven temperature gradient was set to 100°C (0 min hold), with a 12.5°C/min increase to 300°C, followed by a 30°C/min increase to 320°C (2 min hold). The MS was operated in the time-shared SIM mode whereby closely eluting amino acids were analysed in SIM blocks. Based on the well characterised EI fragmentation patterns of TBDMS derivatized molecules (see: National Institute of Standards and Technology (NIST) database) and [5]), the following ions were monitored as detailed in Table 2.

Table 2. TBDMS derivatized amino acids monitored by EI GC-MS

Metabolite	Retention Time (min)	Base Ion (m/z)	Internal Standard Ion (m/z)
Alanine	7.59	158	161
Glycine	7.83	246	249
Valine	8.64	186	191
Urea	8.66	231	234
Leucine	8.98	274	280
Isoleucine	9.27	274	280
Proline	9.67	184	189
Methionine	11.27	218	223

Serine	11.37	390	394
Threonine	11.61	404	409
Phenylalanine	12.24	234	243
Aspartate	12.62	418	423
Glutamate	13.46	432	438
Lysine	14.20	329	336
Glutamine	14.50	431	436
Arginine	14.90	442	451
Histidine	15.57	440	449
Tyrosine	15.86	302	305
Cystine	18.78	348	352

Chromatograph peaks were integrated using the Quantitative Mass Hunter Workstation. Absolute plasma urea and amino acid concentrations were calculated from linear regression of the serially diluted external (unlabeled) standards using the isotope dilution technique. As we did not have isotope labelled internal standards for asparagine and tryptophan, these amino acids were omitted from analysis.

Plasma β-hydroxybutyrate analysis

Plasma β-hydroxybutyrate concentrations were determined using the same EI GC-MS instrumentation and settings as above for amino acids and urea. Briefly, 20 µL of plasma, serially diluted unlabelled β-hydroxybutyrate (D-3-hydroxybutyrate) external standards (0-1 mM; Sigma-Aldrich; 54920-1G-F), and extraction blanks (water) were mixed with 200 µL of methanol containing 25 µM [$^{13}C_4$]sodium D-3-hydroxybutyrate (Cambridge Isotope Laboratories, Inc.; CLM-3853-0.25) internal standard. Samples were vortexed and centrifuged at 15,000 rpm for 10 min at 4°C after which 160 µL of the supernatant was transferred into 250 µL glass GC inserts and evaporated in a speed vacuum at 37°C. Samples were then derivatized by adding 25 µL of pyridine (Sigma-Aldrich) and 25 µL of N,O-bis(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane (Sigma-Aldrich) followed by incubation at 60°C for 30 min. Samples were injected (1 µL splitless; 50 mL/min purge

flow; 0.5 min purge time) with the trimethylsilyl β-hydroxybutyrate derivative analysed by SIM of the 233 m/z (naturally occurring; M0) and 237 m/z (internal standard; M+4) ions, with a 10 ms dwell time per ion. Chromatograph peaks were integrated using the Quantitative Mass Hunter Workstation. Absolute plasma β-hydroxybutyrate concentrations were calculated from linear regression of the serially diluted external (unlabeled) standards using the isotope dilution technique.

Plasma glycerol analysis

Plasma glycerol concentrations were determined via GC-MS using the glycerol triacetate derivative [6]. Briefly, 20 μL of plasma, serially diluted unlabeled glucose standards (0-500 μM) and extraction blanks (water) were mixed with 200 μL of methanol containing 10 μM [$^{13}C_3D_5$]glycerol (Cambridge Isotope Laboratories, Inc.) internal standard. Samples were vortexed and centrifuged at 15,000 rpm for 10 min at 4°C after which 160 μL of the supernatant was transferred into 250 μL glass GC inserts (Agilent Technologies) and dried in a speed vacuum at 37°C. Samples were then derivatized by adding 25 μL of pyridine (Sigma-Aldrich) and 25 μL of acetic anhydride (Sigma-Aldrich) followed by incubation at 60°C for 30 min. To remove excess unreacted acetic anhydride while preventing loss of the volatile glycerol triacetate derivative, 75 μL of methanol was added to each sample without drying in a speed vacuum followed by incubation at 70°C for 15 min, thus reacting all remaining acetic anhydride to form unreactive methyl acetate, after which samples were ready for GC-MS analysis via positive chemical ionization using the same instrumentation as described above for glucose analysis. Glycerol triacetate was analysed via SIM of the M0 (naturally occurring glycerol; 159 m/z) and M+8 ([$^{13}C_3D_5$]glycerol; 167 m/z) ions with a 10 ms dwell time for each. Samples (5 μL) were injected in the splitless mode (50 mL/min purge flow; 0.5 min purge time). The ion abundances were determined using the Quantitative Mass Hunter Workstation (Agilent

Technologies) and absolute plasma glycerol concentrations were calculated from linear regression of the serially diluted external (unlabeled) standards using the isotope dilution technique.

Calculation of glucose fluxes

Glucose fluxes were modelled using the non-steady state equations of Steele (see below), whereby the volume of distribution (V) and pool fraction (*p*) were assumed to equal 200 ml/kg and 0.65 respectively [7, 8], and Inf refers to the [6,6-^{2}H]glucose tracer infusion rate, G_{end1} and G_{end2} are endogenous glucose concentrations (mM) at times t_1 and t_2, TTR_1 and TTR_2 are the ratios between absolute [6,6-^{2}H]glucose tracer concentrations (mM) and G_{end} at times t_1 and t_2:

$$\mathrm{EGP} = \left(\frac{\mathrm{Inf} - \left[\mathrm{pV}\left(\mathrm{G}^{\mathrm{end1}} + \mathrm{G}^{\mathrm{end2}}\right)/2 \times \left(\mathrm{TTR}^{2} - \mathrm{TTR}^{1}\right)/\left(\mathrm{t}^{2} - \mathrm{t}^{1}\right)\right]}{\left(\mathrm{TTR}^{2} - \mathrm{TTR}^{1}\right)/2}\right) - \mathrm{Inf}$$

The rate of glucose disposal (Rd) was calculated by the following equation:

$$\mathrm{Rd} = (\mathrm{EGP} + \mathrm{Inf}) - \mathrm{pV}\frac{\left(\mathrm{G}^{2} - \mathrm{G}^{1}\right)}{\left(\mathrm{t}^{2} - \mathrm{t}^{1}\right)}$$

where G represents the total plasma glucose concentration (tracee + tracer) at times t_1 and t_2. Curve fitting of the change in glucose mass over time was used to improve accuracy of the model [7]. Total glucose production per subject was calculated via integration of the EGP rate (μmol/kg/min) over the experimental period (0-240 min AUC) multiplied by bodyweight.

Special note: plasma glucagon assay

Given the well documented problems with measuring plasma glucagon concentrations via immunoassays due to cross-reactivity with other related glucagon gene products (e.g. the proglucagon fragments glicentin and oxyntomodulin [9]), we used the commercially available

glucagon ELISA from Mercodia due to its extensive validation including comparisons to quantification via isotope dilution tandem mass spectrometry [10-15].

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