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The second-meal phenomenon is associated with enhanced muscle glycogen storage in humans

Ana JOVANOVIC∗†, Emily LEVERTON‡, Bhavana SOLANKY‡, Balasubramanian RAVIKUMAR∗†, Johanna E. M. SNAAR‡, Peter G. MORRIS‡ and Roy TAYLOR∗†

∗Diabetes Research Group, Institute of Cellular Medicine, Newcastle University, Newcastle upon Tyne NE4 5PL, U.K., †Newcastle Magnetic Resonance Centre, Institute of Cellular Medicine, Newcastle University, Newcastle upon Tyne NE4 5PL, U.K., and ‡Magnetic Resonance Centre, School of Physics and Astronomy, University of Nottingham, Nottingham NG7 2RD, U.K.

ABSTRACT

The rise in blood glucose after lunch is less if breakfast has been eaten. The metabolic basis of this second-meal phenomenon remains uncertain. We hypothesized that storage of ingested glucose as glycogen could be responsible during the post-meal suppression of plasma NEFAs (non-esterified fatty acids; ‘free’ fatty acids). In the present study we determined the metabolic basis of the second-meal phenomenon. Healthy subjects were studied on two separate days, with breakfast and without breakfast in a random order. We studied metabolic changes after a standardized test lunch labelled with 3 g of 13C-labelled (99 %) glucose. Changes in post-prandial muscle glycogen storage were measured using 13C magnetic resonance spectroscopy. The rise in plasma glucose after lunch was significantly less if breakfast had been taken (0.9 ± 0.3 compared with 3.2 ± 0.3 mmol/l, with and without breakfast respectively; P < 0.001), despite comparable insulin responses. Pre-lunch NEFAs were suppressed after breakfast (0.13 ± 0.03 compared with 0.51 ± 0.04 mmol/l) and levels correlated positively with the maximum glucose rise after lunch (r = 0.62, P = 0.001). The increase in muscle glycogen signal was greater 5 h after lunch on the breakfast day (103 ± 21 compared with 48 ± 12 units; P < 0.007) and correlated negatively with plasma NEFA concentrations before lunch (r = −0.48, P < 0.05). The second-meal effect is associated with priming of muscle glycogen synthesis consequent upon sustained suppression of plasma NEFA concentrations.

INTRODUCTION

The extent of post-prandial rise in plasma glucose depends not only upon the quantity and nature of food ingested, but also upon the metabolic state immediately prior to eating. In a previous study we made the incidental observation that the rise in blood glucose was considerably less after the second of two similar meals [1]. On searching the literature it became clear that this had first been described almost a century ago as the second-meal phenomenon or Staub–Traugott effect [2,3]. Despite renewed interest in the phenomenon several decades ago [4–7], the mechanism underlying this effect remains unknown.

Key words: glucose, glycogen, magnetic resonance spectroscopy, non-esterified fatty acid (‘free’ fatty acid), second-meal phenomenon, Type 2 diabetes mellitus.

Abbreviations: APE, atom percentage excess; CV, coefficient of variation; MR, magnetic resonance; MRS, MR spectroscopy; NEFA, non-esterified fatty acid (‘free’ fatty acid).

Correspondence: Professor Roy Taylor (email Roy.Taylor@ncl.ac.uk).
Limiting the rise in plasma glucose after meals is important, as the extent of the post-prandial rise in blood glucose is a risk factor for cardiovascular disease independent of fasting plasma glucose [8–11]. The relationship applies within the normal range of glucose values as well as in impaired glucose tolerance and diabetes. Considerable effort is being directed towards development of therapeutic agents which specifically target post-prandial hyperglycaemia [12–14]. However, there is a potential health advantage to the whole population of being able to understand the second-meal phenomenon as a physiological means of minimizing the rise in blood glucose concentration after eating.

The present study was undertaken to establish whether the second-meal effect was a consequence of enhanced rates of glycogen synthesis in muscle secondary to post-first-meal suppression of plasma NEFAs (non-esterified fatty acids; ‘free’ fatty acids). The development of $^{13}$C MRS [MR (magnetic resonance) spectroscopy] has allowed direct, non-invasive quantification of glycogen storage.

MATERIALS AND METHODS

Subjects

Healthy subjects on no medication were recruited. Ethical permission was obtained from the Newcastle and North Tyneside Local Research Ethics Committee. Written informed consent was obtained from each subject before the start of the study. In total, ten subjects were recruited [mean age, 46.7 $\pm$ 3.9 years; weight, 76.3 $\pm$ 3.1 kg; and BMI (body mass index), 26.1 $\pm$ 1.1 kg/m$^2$].

Study design

Subjects were studied on two separate days in a random order. On one day subjects consumed a standard breakfast followed by a standard lunch including $^{13}$C-labelled glucose 4 h later. On the other day breakfast was omitted. Subjects abstained from alcohol or vigorous exercise for 3 days before each study and followed their habitual diet. Subjects fasted from 18.00 hours on the evening before the study. In total, ten subjects were recruited [mean age, 46.7 $\pm$ 3.9 years; weight, 76.3 $\pm$ 3.1 kg; and BMI (body mass index), 26.1 $\pm$ 1.1 kg/m$^2$].

Protocol

On the morning of the study, subjects were transported to the Magnetic Resonance Centre by taxi. A venous cannula was placed in the dorsum of one hand. This hand was kept in a heated box at 55 $^\circ$C to allow sampling of arterialized blood, and during periods in the scanner the hand was kept at the same temperature using a heat-retaining kaolin pack. The subject was positioned in the scanner and fasting muscle glycogen was measured. The baseline blood samples were then taken and the standard breakfast was provided. On the breakfast day, time zero was set as the commencement of the standard breakfast. On the day without breakfast the subjects fasted until lunchtime but otherwise the protocol was the same. Repeat glycogen measurements were taken at +2 and +4 h. At +4 h, the subjects were given the standard lunch which incorporated 3 g of [U-$^{13}$C]glucose. Muscle glycogen was subsequently measured at +6 and +9 h. Subjects were asked to eat the test meals at their normal rate. Both breakfast and lunch meals were consumed within 15 min in all cases. Subjects remained in a sitting position (except for the standardized supine periods in the magnet) to avoid postural differences in the rate of gastric emptying between test days. Arterialized blood samples were collected hourly. Breath samples for $^{13}$CO$_2$ were obtained before the labelled lunch and at 1-h intervals thereafter.

Meal composition

The standard breakfast consisted of 50 g of muesli, 100 g of milk, 2 slices of toast (56 g), 20 g of marmalade, 20 g of margarine and 200 ml of orange juice [106 g of carbohydrate, 18 g of fat and 15 g of protein; 646 kcal (1 kcal $\approx$ 4.184 kJ)].

The standard lunch comprised a cheese sandwich, 200 ml of orange juice and 170 g of yoghurt and jelly (103 g of carbohydrate, 30 g of fat, 44 g of protein; 858 kcal). A 100 ml jelly contained 3 g of [U-$^{13}$C]glucose (atom 99 %; Cambridge Isotopes) and was divided into four cubes and eaten at equally timed intervals during the meal to allow even dispersion throughout the gastric contents. Subjects were asked to chew each cube thoroughly before swallowing.

Breath $^{13}$C enrichments

Breath samples for $^{13}$C enrichments were collected at intervals. The subject was asked to blow through a straw into a glass tube. This was immediately capped with a rubber top at the end of a full exhalation. $^{13}$C enrichments of breath samples were determined by continuous flow isotope ratio MS [ABC system; PDZ Europa; CV (coefficient of variation) for the analysis, 0.07 % and CV for the collection, 0.3 %]. All results of the $^{13}$C enrichment of expired air are expressed as APE (atom percentage excess).

Blood glucose, plasma insulin and metabolites

Blood glucose was measured with a HemoCue photometer analyser. Plasma NEFAs were measured on a Roche Cobas centrifugal analyser (Roche Diagnostics) using a commercially available enzymatic colorimetric kit (Wako Chemicals). Serum insulin and C-peptide were both measured using ELISA kits (Dako). The plasma glucagon concentration was measured by radioimmunoassay (Linco Research). Metabolites (glycerol, lactate, pyruvate, $\beta$-hydroxybutyrate and alanine) were measured using a Cobas bio centrifugal analyser. Plasma triacylglycerols (triglycerides) were measured on a Roche
Cobas centrifugal analyser, using a colorimetric assay (ABX Diagnostics). Plasma catecholamines were measured using an enzyme immunoassay (Labor Diagnostica).

**13C MRS and quantification of spectra**

MR data were acquired on a 3 T whole-body MR scanner. Muscle glycogen measurements were taken from the quadriceps muscle using a surface coil comprising a 7 cm diameter carbon coil for transmission and reception and quadrature proton coils for 1H decoupling. A small vial, containing [13C]formate and fixed at the centre of the 13C coil, was used as a reference for determining glycogen concentrations. The subject was placed in a comfortable supine position and the coil was placed directly over the mid-anterior aspect of the thigh and held firmly in place with a Velcro strap. The area was marked to allow the coil to be repositioned accurately.

Manual shimming was performed on the water resonance peak, and the broadband decoupling frequency was centred on the glycogen resonance. The coils were tuned and matched before each measurement using a network analyser (HP model 8751A, 5–500 KHz). The 13C spectra were acquired using a pulse-acquire sequence with proton decoupling. The excitation pulse was a 100 µs hard pulse at a peak power of 390 W with CYCLOPS phase cycling. Broadband decoupling was achieved using the WALTZ-8 sequence during signal acquisition with a peak power of 50 ± 2 W. A repetition interval of 360 ms was used and 3000 acquisitions were averaged for each spectrum giving a temporal resolution of 18 min. RF (radio frequency) power was monitored throughout the acquisition period to ensure that it did not exceed the maximum value allowed according to the specific absorption rate limits recommended by the National Radiological Protection Board.

The spectra were processed using the Matlab version of the MRUI (MR user interface). The integral of the glycogen peak was expressed as a fraction of the integral of the formate peak arising from the vial containing [13C]formate at the centre of the 13C coil. Quantification was achieved by comparison with a phantom containing 184 mmol/l oyster glycogen and 150 mmol/l KCl. The concentration of muscle glycogen, [Glyc]m, was calculated using the formula:

$$[\text{Glyc}]_m = \frac{R_\text{muscle} \times [\text{Glyc}]_\text{phantom}}{R_\text{phantom}}$$

where $R_\text{phantom}$ and $R_\text{muscle}$ are the ratios of the integrals of the glycogen-to-formate peaks in the phantom and muscle respectively, and $[\text{Glyc}]_\text{phantom}$ is the concentration of glycogen in the phantom (184 mmol/l).

Before lunch the [13C]glycosyl units were at a natural abundance and the rise in absolute concentrations can be determined as the glycogen in mmol/l of muscle. After the lunch containing [13C]glucose, the percentage enrichment of the glycosyl units in muscle glycogen is no longer known, and glycogen concentration is reported as the total signal intensity in units/l.

The CV for 13C MRS muscle glycogen measurement has previously been shown to be 4.3 ± 2.1 % compared with 9.3 ± 5.9 % by biopsy measurement [15].

**Statistical analysis**

Values are presented as means ± S.E.M. All statistical calculations were performed using Minitab software (Release 15). Comparisons were carried out using a two-tailed paired Student’s $t$ test, and relationships were tested using the linear correlation analysis. Statistical significance was accepted at $P < 0.05$. A prior power calculation identified that, to detect a 40 % change in muscle glycogen concentration with 95 % power, eight subjects had to be studied.

**RESULTS**

**Glucose**

On the breakfast day, blood glucose rose from 4.5 ± 0.2 mmol/l to a peak at 7.8 ± 0.3 mmol/l at 1 h after breakfast, returning to 5.0 ± 0.2 mmol/l before lunch (Figure 1). After lunch, the peak post-prandial glucose was lower than after breakfast (6.1 ± 0.3 mmol/l, $P < 0.001$) and the increment was considerably less (0.9 ± 0.3 compared with 3.3 ± 0.3 mmol/l, $P < 0.001$). On the day without breakfast, blood glucose fell from 4.7 ± 0.1 to 4.4 ± 0.2 mmol/l during the prolonged fast before lunch. The glucose level at 1 h after the lunch was significantly greater compared with that on the breakfast day (7.6 ± 0.3 compared with 5.9 ± 0.2 mmol/l, $P < 0.004$). The difference remained significant when the glucose increments were compared (3.2 ± 0.3 compared with 0.9 ± 0.3 mmol/l, $P < 0.001$).

**Serum insulin and C-peptide**

On the breakfast day, serum insulin rose from 4.5 ± 0.4 m-units/l to a peak level of 63.5 ± 10.4 m-units/l at 1 h
Changes in serum insulin, C-peptide and glucagon during the study period on days with (A) and without (B) breakfast.

Values are means ± S.E.M.

After breakfast and fell to 18.0 ± 4.2 m-units/l at lunchtime. The post-lunch insulin peak was 64.5 ± 9.3 m-units/l 30 min later with a slow return to the baseline at the end of the study. On the day without breakfast insulin levels declined slowly until lunch (5.5 ± 0.8 m-units/l at 0 h and 3.4 ± 0.5 m-units/l at 4 h, \( P = 0.002 \)). Serum insulin peaked at 68.1 ± 10.7 m-units/l at 1 h after lunch (\( P = 0.72 \) compared with the breakfast day at 30 min after lunch). Subsequently, insulin levels remain higher on the day without breakfast (25.4 ± 6.1 compared with 16.3 ± 4.0, \( P < 0.05 \) (Figure 2A.).

The change in C-peptide levels mimicked the change in insulin levels. On the breakfast day C-peptide levels rose to a maximum of 3.3 ± 0.3 nmol/l at 1 h after breakfast, returning to 2.0 ± 0.3 nmol/l before lunch. C-peptide rose back to 3.3 ± 0.4 nmol/l at 1 h after lunch and slowly declined from then on. On the day without breakfast, C-peptide concentrations peaked at 3.0 ± 0.3 nmol/l at 1 h and were still elevated by the end of the day (Figure 2B).

### Glucagon and catecholamines

Mean fasting glucagon levels were similar on the two test days (52.7 ± 4.5 pg/ml and 57.6 ± 6.2 pg/ml respectively). Glucagon levels were stable until lunch on both days and gradually increased towards the end of the study days (Figure 2C).

Immediately before lunch, plasma noradrenaline levels were similar on the two study days (1.51 ± 0.18 and 1.47 ± 0.19 nmol/l) with no further change 30 min after lunch. The pre-lunch mean plasma adrenaline concentration was slightly higher on the day without breakfast (prolonged fast; 0.37 ± 0.04 compared with 0.27 ± 0.01 nmol/l, \( P < 0.05 \)). Post-lunch, plasma adrenaline decreased to 0.33 ± 0.03 nmol/l (\( P = 0.07 \)) on the day without breakfast, remaining constant on the breakfast day (0.27 ± 0.01 and 0.28 ± 0.01 nmol/l).

### Plasma NEFAs, triacylglycerols and metabolites

Plasma NEFAs were suppressed rapidly on the breakfast day, reaching a nadir at +2 h (basal, 0.48 ± 0.04 nmol/l; 2 h, 0.08 ± 0.01 nmol/l), and remaining suppressed until the end of the study day. On the day without breakfast, plasma NEFAs remained elevated until 1 h after lunch, falling to a nadir at +6 h (0.09 ± 0.01 nmol/l) with no further change until the end of the study day (Figure 3).

Plasma NEFA concentrations pre-lunchtime correlated positively with the maximum glucose rise after lunch (\( r = 0.70, P < 0.001 \); Figure 4).

Glycerol and \( \beta \)-hydroxybutyrate levels are shown in Figure 3. Both decreased rapidly after the first meal of the day. Plasma lactate, pyruvate and alanine showed the expected rise in concentration after meals as the splanchnic tissues switched from lactate uptake to release. Most of the lactate originates from the liver, whereas the gut contributes the majority of circulating alanine, based on previous studies in the conscious dog [15a].

Fasting triacylglycerol levels were comparable on the two test days (1.27 ± 0.19 and 1.28 ± 0.20 mmol/l). On the breakfast day, triacylglycerol levels rose steadily during the course of the study day, reaching levels significantly greater from baseline and peaking at +6 h (2.09 ± 0.26 mmol/l, \( P < 0.001 \)). On the day without breakfast, triacylglycerol levels started to rise from 1.08 ± 0.15 mmol/l at 1 h after lunch to a peak of 1.66 ± 0.27 mmol/l at the end of the study day.

### Muscle glycogen

Mean fasting glycogen concentrations were similar on the two days (60 ± 8 compared with 63 ± 12 mmol/l,
Figure 3 Changes in plasma NEFAs, glycerol, β-hydroxybutyrate (BOH), pyruvate, alanine and lactate during the study period on days with (■) and without (○) breakfast.
Values are means ± S.E.M.

On the day without breakfast, the mean fasting glycogen concentration remained stable until lunch (63 ± 13 mmol/l at 0 h and 59 ± 13 mmol/l at +4 h). Following lunch there was a slower rise in the muscle glycogen signal intensity to 98 ± 16 units/l at 2 h and 105 ± 18 units/l at the end of the study day. The increment over baseline was significantly greater on the breakfast day at the end of the study day (103 ± 21 compared with 48 ± 12 units/l, P < 0.007; Figure 5A). There was an inverse correlation between this increase in muscle glycogen signal and plasma NEFA concentrations before lunch (r = −0.48, P < 0.05; Figure 5B).

Breath 13C enrichments
The appearance of 13C in expired breath was similar on days with and without breakfast. 13C APE steadily increased to a maximum at 4 h after lunch with values of 0.81 ± 0.06 compared with 0.76 ± 0.06 (P = 0.36) on the two study days (Figure 5C).

DISCUSSION
The present study has demonstrated for the first time that the second-meal phenomenon is associated with increased glycogen synthesis in skeletal muscle in humans. The rate of incorporation of dietary glucose into muscle glycogen after lunch is approx. 50% greater within 2 h and approx. doubled within 5 h when breakfast had been taken compared with no-breakfast days. The
meal-induced insulin response was similar on the two days. The plasma NEFA concentration before lunch correlated positively with the post-lunch rise in blood glucose, and negatively with the increase in the glycogen signal after lunch. Our results suggest that insulin secretion after breakfast suppresses plasma NEFAs, facilitating carbohydrate economy after lunch and permitting greater storage of glycogen in muscle. An additional direct effect of recent exposure of muscle to insulin cannot be excluded [16]. The conclusions of the study may be of practical benefit to athletes wishing to maximize performance and to people with Type 2 diabetes mellitus, a condition in which muscle glycogen synthesis is impaired. Furthermore, the response of counter-regulatory hormones glucagon, adrenalin and noradrenalin following the second meal were similar.

The present study was triggered by our previous incidental observation of a 70 % lesser rise in blood glucose after lunch than after a similar meal at breakfast time [1]. However, this could have been at least partially an effect of the diurnal cortisol profile. In the present study we examined the rise in blood glucose only at lunchtime thus avoiding this complicating factor. As lunch, when given after a breakfast rather than after no breakfast, was associated with a 73 % reduction in blood glucose increment, the major influence is established as the second-meal phenomenon rather than a time-of-day effect. Although this was first observed almost a century ago [2,3], the underlying mechanism has not been elucidated. Re-discovery of the phenomenon decades later [4,5] was followed by a series of investigations upon anaesthetized animals using intravenous glucose administration [6,17]. These studies showed no effect of a prior dose of glucose on glycogen storage in liver or muscle, nor on glucose oxidation, but the effects observed are likely to have been affected by increasing duration of anaesthetic. By investigating the second-meal phenomenon in humans, direct observation of the effect upon muscle glycogen storage as glycogen and meal-derived glucose oxidation was possible.

The second meal is given after the body has already taken in energy and it may be considered that the greater total carbohydrate intake would lead to increased glycogen stores later in the day. However, the study design of using a \[^{13}\text{C}\]-labelled lunch allows storage of glycogen derived from carbohydrate in the lunch to be assessed as the detected increment in muscle glucose. The breakfast carbohydrate itself is not likely to contribute to the increase glycogen storage in the period after lunch, as storage of the first-meal carbohydrate as glycogen is complete within the first 5 h [18]. Hence, there would be a negligible contribution of breakfast carbohydrate to the observed increase in \[^{13}\text{C}\]glycogen concentration 2 and 5 h after lunch, as glycogen derived from breakfast would not only be at a natural abundance but also would be very small in absolute amount. The loss of possibility of interpreting signal change as representing total muscle glycogen is a necessary and small price to pay for the definitive tracing of lunch-derived glucose.

Non-invasive MRS has been shown to measure muscle glycogen concentration accurately and precisely in comparison with biopsy [15]. Using this technique we have previously demonstrated that storage of glucose as muscle glycogen accounts for 30 % of ingested carbohydrate at peak, 5 h after the test meal in healthy subjects [19]. Thereafter the glycogen concentration in muscle was observed to fall. Total glucose oxidation has been observed to account for approx. 50 % of ingested...
carbohydrate over the same period [18]. Modulation of either or both of these processes is capable of changing the plasma glucose rise after eating. When two successive meals are taken, muscle glycogen concentration rises in a stepwise fashion after each meal [1]. Muscle glycogen is in a state of continuous synthesis and degradation in the fasting state [20] and during gentle exercise [21]. However, in the insulin-stimulated state after a meal, muscle glycogen breakdown is minimized and the glucose stored as glycogen will reflect the \([^{13}C]\)glucose APE in plasma [22]. As only the lunch was isotopically enriched, the post-lunch marked rise in glycogen concentration reflects uptake and storage as glycogen of ingested carbohydrate. The results of the present study demonstrate a clear effect of a meal taken 4 h previously upon uptake of meal-derived glucose and storage as muscle glycogen. The question arises as to the underlying mechanism for modulation of glycogen synthesis, and the inverse relationship of plasma NEFA concentration to glycogen. The possibility of some direct effect of prior ingestion of carbohydrate over the same period [18]. The question arises as to the underlying mechanism for modulation of glycogen synthesis, and the inverse relationship of plasma NEFA concentration to glycogen. The possibility of some direct effect of prior ingestion of carbohydrate over the same period [18]. The question arises as to the underlying mechanism for modulation of glycogen synthesis, and the inverse relationship of plasma NEFA concentration to glycogen. The possibility of some direct effect of prior ingestion of carbohydrate over the same period [18]. The question arises as to the underlying mechanism for modulation of glycogen synthesis, and the inverse relationship of plasma NEFA concentration to glycogen. The possibility of some direct effect of prior ingestion of carbohydrate over the same period [18].

In the 1980s there was considerable interest in the effect of low-glycaemic index breakfasts in improving the post-lunch blood glucose profile [32,33]. This effect on the total area under the glycaemic response curve to lunch was subsequently shown to relate directly to the concentration of plasma NEFAs at the time of lunch [34]. If the quantity of carbohydrate consumed at breakfast was insufficient to suppress plasma NEFAs then no effect of glycaemic index of breakfast upon post-lunch glycaemia was observed [35]. To preserve the second-meal phenomenon throughout the day it would appear necessary that meals or snacks are taken frequently enough to maintain suppression of plasma NEFAs and hence perpetuate the post-prandial state.

Application of such dietary manipulation could have considerable implications in people with diabetes. However, one study has suggested that the second-meal phenomenon does not occur in diabetes [35]. Clark et al. [35] used intravenous glucose loads in diabetic subjects with very poor insulin secretory response and, although not measured, it is probable that NEFA suppression was not achieved. We have recently demonstrated that the second-meal phenomenon is potently expressed in people with Type 2 diabetes mellitus when day-to-day conditions are reproduced using mixed meals [36].

These observations raise the possibility that mimicking the second-meal phenomenon could be utilized to limit the relatively large rise in blood glucose seen after the first meal of the day in people with impaired glucose tolerance or Type 2 diabetes mellitus. This could be achieved by taking a low-carbohydrate, high-protein snack to bring about insulin secretion and NEFA suppression pre-breakfast or by pharmacological reduction of pre-breakfast plasma NEFAs. The minimal size of the first meal to achieve adequate suppression of plasma NEFAs is now being investigated.

In summary, the second-meal phenomenon is associated with enhanced muscle glycogen storage and appears to be determined by suppression of plasma NEFAs. These findings have implications for controlling excessive swings in post-prandial glycaemia by therapeutic application of the second-meal effect.

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