Time course of fractional gluconeogenesis after meat ingestion in healthy adults: a D$_2$O study

Claire Gaudichon, Hai-Yen Ta, Nadezda V. Khodorova, Marion Oberli, Isabelle Breton, Robert Benamouzig, Daniel Tomé, and Jean-Philippe Godin.

INTRODUCTION

Gluconeogenesis is a key pathway in the regulation of glucose homeostasis in overnight and postabsorptive conditions. In fasting conditions, liver is the main source of glucose from two distinct pathways of gluconeogenesis and glycogenolysis (23, 28) and their relativity to liver endogenous glucose production (EGP) depends on the metabolic and nutritional status of the individual (2, 25). It is generally accepted that in healthy individuals, these pathways contribute equally to glucose production. As the fasting period progresses, glycogen stores deplete, and gluconeogenesis then becomes the main source of glucose during this postprandial phase and that the decrease of glucose production (11). We previously showed that after a protein/lipid meal ingested as an omelet, EGP remained stable for 5 h after the meal and subsequently decreased and that dietary amino acids accounted only for 10% of this production (9). This suggested that glycogen was the main source of glucose during this postprandial phase and that the decrease of EGP corresponded to the exhaustion of liver glycogen. However, the protocol did not permit measuring fractional gluconeogenesis. In the present study, we aimed at quantifying the evolution of fractional gluconeogenesis after a meat meal. Thirteen healthy subjects received oral doses of D$_2$O. After fasting overnight, they ingested a steak (120 g). Glycemia, insulinemia, and D$_2$O enrichments in glucose and plasma water were measured for 8 h after the meal. Fractional gluconeogenesis was assessed using the average method. Glucose was stable for 5 h and then decreased. There was a slight increase of insulin 1 h after the meal. D$_2$O enrichment in the carbon 5 position of glucose (C5) increased after 2 h, whereas it decreased in plasma water. Consequently, fractional gluconeogenesis increased from 68.2 ± 7.2% before the meal to 75.5 ± 5.8% 8 h after the meal, the latter corresponding to 22 h without a glucose supply. These values are consistent with the exhaustion of glycogen stores after 24 h but represent the highest among values in the literature. The impact of methodological conditions is discussed.

average method; deuterated water; glucose homeostasis; postprandial gluconeogenesis; protein ingestion

The regulation of gluconeogenesis is based on enzymatic regulations in the liver, depending on the availability of gluconeogenic precursors from diet and endogenous sources. Several studies have looked at the role of glutamine (22), lactate (14), glycerol (29), galactose (10), and fructose (19) in gluconeogenesis and showed that there are minor changes to gluconeogenesis when the availability of such substrates increases. The influence of protein meals or diet has been questioned regarding its impact on glucose homeostasis and, more specifically, gluconeogenesis regulation. Protein meals have been assumed to enhance gluconeogenesis (31). Moreover, high-protein diets increase protein turnover, leading to an enhancement of amino acid release from body protein breakdown, which has been suspected to be involved in the increased risk of diabetes (18). Nevertheless, protein or amino acid ingestion does not enhance glucose endogenous production (11). We previously showed that after a protein/lipid meal ingested as an omelet, EGP remained stable for 5 h after the meal and subsequently decreased and that dietary amino acids accounted only for 10% of this production (9). This suggested that glycogen was the main source of glucose during this postprandial phase and that the decrease of EGP corresponded to the exhaustion of liver glycogen. However, the protocol did not permit measuring fractional gluconeogenesis. In the present study, we aimed at quantifying the evolution of fractional gluconeogenesis after ingestion of a protein meal (as meat) using oral ingestion of deuterium water (D$_2$O).

Techniques used to measure fractional gluconeogenesis are still diverse and have been reviewed in detail in various publications (8, 24). The method based on D$_2$O administration developed by Landau et al. (17) is considered the most accurate but requires many analytical steps to generate hexamethylenetetramine (HMT) before its analysis by gas-chromatography-mass spectrometry (GC-MS). In 2008, Chacko et al. (6) published a new GC-MS method (named “average method”) to assess fractional gluconeogenesis after administration of deuterated water without the tedious chemical transformation of glucose into HMT. Our objective was to quantify fractional gluconeogenesis kinetics after meat intake in healthy adults using the average method. Based on the previous study where we assessed EGP after egg intake, we hypothesized that without any dietary glucose supply, the fractional gluconeogenesis after meat consumption would slightly increase.

Address for reprint requests and other correspondence: C. Gaudichon, UMR PNCA, AgroParisTech, 16 rue Claude Bernard, 75231 Paris, France (e-mail: claire.gaudichon@agroparistech.fr).
Table 1. Subject characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex, M/F</td>
<td>7/6</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>22.9 ± 3.2</td>
</tr>
<tr>
<td>Age, y</td>
<td>32.5 ± 9.0</td>
</tr>
<tr>
<td>Body water, kg</td>
<td>M: 42.6 ± 2.6, F: 31.0 ± 2.1</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>0.47 ± 0.29</td>
</tr>
</tbody>
</table>

BMI, body mass index; F, female; HOMA-IR, homeostasis model assessment of insulin resistance; M, male.

MATERIALS AND METHODS

Subjects. The intervention study has been previously described in detail (21). It was primarily designed to measure ileal digestibility of meat proteins according to two cooking processes. A secondary objective was to measure fractional gluconeogenesis after meat ingestion. The study was approved by the Ethical Committee of St-Germain-en-Laye Hospital (St-Germain-en-Laye, France) and was registered at https://clinicaltrials.gov/ (NCT01685307). The eligibility criteria were: negative serology for HIV, hepatitis B virus surface antigen, and hepatitis C; absence of any pathology; absence of pregnancy or contraception for women; body mass index between 18 and 30; and aged between 18 and 50 yr. Among the 24 volunteers included in the study, 13 were randomly selected for assessment of fractional gluconeogenesis after meat ingestion. Their characteristics are presented in Table 1.

Protocol. The day before the postprandial investigation, subjects were given 3 doses of D₂O enriched at 99% ²H (Eurisotop, St Aubin, France) at 1-h intervals (19:00, 20:00, and 21:00) to reach a final dose of 5 g/kg of body water, as previously described (6, 30). Body water was calculated using Watson equations. Subjects were fasted overnight, and the next morning a catheter was inserted in a forearm vein. After blood sampling, subjects ingested the test meal consisting of 120 g of bovine meat. They were given ~100 ml of water hourly, enriched at 0.5% with deuterium to maintain an isotopic steady state (16).

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By design, no baseline (preadministration of D₂O) blood samples were collected. To estimate the excess of isotopic enrichment expressed in molar percent excess (MPE), we used anonymized plasma samples from Biopredic International (St-Gregoire, France).

Analyses. Plasma glucose was assayed by a glucose oxidase method (Glucose RTU kit, BioMérieux, Lyon, France). Plasma insulin was determined using an endocrine kit (Bio-Plex ProTM Assay, Bio-Rad Laboratories, Hercules, CA) in a Bioplex 200 system (Bio-Rad Laboratories).

Fractional gluconeogenesis was assessed using the average method based on determination of the ²H isotopic enrichment of carbons 1, 3, 4, 5, and 6 of the glucose derivative to get the average deuterium isotopic enrichment of the glucose derivative and by measuring body water deuterium isotopic enrichment from the plasma sample (6). Plasma samples (25 and 80 μl) were used for the average deuterium glucose isotopic enrichment and for plasma body water isotopic enrichment determination, respectively. Deuterium isotopic enrichment of the glucose derivative was measured by GC-MS (GC 6890, MS 5973N; Agilent Technology, Germany) using pentaacetate derivative in positive chemical ionization mode. A selective ion monitoring mode of m/z 170/169 was monitored to assess the deuterium enrichment of the glucose derivative. In each analytical sequence, baseline plasma samples were measured and the M+1/M ratio (or m/z 170/169) obtained was 0.09194 ± 0.00067 (n = 7 days).

Deuterium isotopic enrichment of body water from plasma was determined by Total Conversion-Isotope Ratio Mass Spectrometer measurement, the delta values generated by the mass spectrometer were normalized to Vienna Standard Mean Ocean Water (V-SMOW) and Standard Light Antarctic Precipitation (SLAP) international values, and the isotopic enrichment was further expressed in MPE. Plasma samples were centrifuged at 4°C at 14,000 g (Ultracentrifuge Heraeus, Thermo Scientific, Germany) for one night in a vial containing a filter with a cut-off value of 5,000 Da (Amicon Ultra, Millipore, Ireland) and then diluted with Milli-Q water (Millipore, Switzerland). Replicate injections were performed for each sample, and only the last four injections were averaged and reported as the isotopic results.

Fractional gluconeogenesis was calculated using deuterium isotopic enrichment of body water (from plasma samples), representing the precursor pool and M+1/M ratio of plasma glucose derivative divided by the number of labile hydrogens in glucose, according to Chacko et al. (6).

Statistics. Values are represented as means ± SD. A primary analysis was done to evaluate whether the group (cooking process) influenced the results. In the absence of any group effect, data were averaged.
pooled. The effect of time was analyzed in a mixed model with time as a repeated factor (SAS 9.1, SAS Institute, Cary, NC). Differences from baseline were analyzed using pairwise post hoc tests adjusted for the number of comparisons.

RESULTS

Subject characteristics. The subjects were healthy adults (46% women) with normal glucose tolerance status (as defined by homeostasis model assessment of insulin resistance, Table 1).

Plasma glucose and insulin response. Fasting glucose concentration was 4.8 ± 0.4 mmol/l (Fig. 1A). After the meat intake, plasma glucose did not significantly increase and was stable for 5 h after the meal, ranging between 5.1 ± 0.6 mmol/l and 5.2 ± 0.4 mmol/l. It then significantly decreased compared with baseline to a nadir value of 4.4 ± 0.4 mmol/l, with a

Fig. 2. Plots of the interindividual variations of the average 2H enrichment of plasma glucose measured by gas-chromatography-mass spectrometry using m/z 170/169 plasma (A), 2H enrichment of water (B), and fractional gluconeogenesis (C) calculated using the average method in 13 subjects after fasting overnight and ingestion of a single meat meal (27 g protein) at t = 0. MPE, molar percent excess.

Fig. 3. Kinetics of the average 2H enrichment of plasma glucose and plasma water 2H enrichment (A) and fractional gluconeogenesis (B) measured in 13 subjects after fasting overnight and ingestion of a single meat meal (27 g protein) at t = 0. Means ± SD. *Significant difference from value at t = 0. MPE, molar percent excess.
significant effect of time (\( P < 0.0001 \)). Insulin concentration (Fig. 1B) significantly increased from baseline to reach a maximum value of 24.6 ± 7.9 pmol/l at 1 h and tended to be lower than baseline after 5 h, with a nadir value of 4.9 ± 2.6 pmol/l at 7 h.

**Fractional gluconeogenesis.** Determination of the fractional gluconeogenesis requires the measurement of deuterium isotopic enrichment in body water used as a precursor pool and the average measurement of deuterium isotopic enrichment in glucose carbon. The results of deuterium kinetics, both in plasma water and in glucose carbon, are reported in Fig. 2 for individual data and Fig. 3 for the mean. Average deuterium enrichment in the glucose derivative ranged between 0.24 and 0.38 MPE among subjects before meat ingestion (Fig. 2A). It significantly increased (time effect: \( P < 0.0001 \)) from 0.29 ± 0.05 MPE before the meal to a maximum value of 0.33 ± 0.04 MPE after 6 h (Fig. 3A). A slight but nonsignificant decrease (\( P = 0.09 \)) was observed after 8 h, to a value of 0.31 ± 0.04 MPE. The deuterium isotopic enrichment of body water ranged between 0.38 and 0.48 MPE among subjects before the meal (Fig. 2B). On average, it slightly but significantly decreased (time effect: \( P = 0.0045 \)) from 0.42 ± 0.03 MPE at basal to 0.41 ± 0.03 MPE at 8 h after meat ingestion (Fig. 3A).

Taken together, these isotopic measurements were combined to estimate the fractional gluconeogenesis (Figs. 2C and 3B). Before meat ingestion, fractional gluconeogenesis ranged between 57% to 79% (Fig. 2C), with an average value of 68.2 ± 7.2%. It progressively increased after the meal to reach a value of 79.3 ± 5.2% at 6 h (time effect: \( P < 0.0001 \)). We observed a slight but not significant (\( P = 0.19 \)) decrease between 6 and 8 h, to a value of 75.5 ± 5.8%.

### DISCUSSION

The present study aimed to determine the kinetics of fractional gluconeogenesis after the ingestion of a meat protein meal using oral D2O administration in healthy adults. We reported a contribution of fractional gluconeogenesis increasing from 68% to 79% at the end of the postprandial investigation (8 h after meat ingestion). To our knowledge, this is the only study that has measured the postprandial evolution of fractional gluconeogenesis after a protein meal.

In this study, subjects fasted overnight for 14 h and then ingested a single meal consisting of meat, providing 27 g protein and 4 g fat. At the end of the postprandial investigation period, subjects had not ingested any glucose source for 22 h. The measured fractional gluconeogenesis found was in accordance with the nutritional state of the subjects, with a minor and decreasing contribution of glycolysis and a minor increase of gluconeogenesis of 2%/h, a value that is consistent with that reported by Katz et al. (15). The results also showed a decrease in blood glucose levels 5 h after ingestion of the protein meal, which is in line with our previous study where we concomitantly observed a decrease of EGP (9). We suggested that it was due to the decrease of glycolysis flux, a hypothesis confirmed by the present results. Chandramouli et al. (7) also showed that the decrease of EGP within 24 h fasting was due to glycolysis, whereas glucose flux remained constant.

In the present study we did not perform any [6,6-2H2]-glucose infusion to determine EGP. As mentioned above, an increase of fractional gluconeogenesis may not be associated with an increase of gluconeogenesis flux when EGP decreases. To further address this question, we extrapolated gluconeogenesis.

### Table 3. Summary of methodological conditions, fasting duration and fractional gluconeogenesis in different studies

<table>
<thead>
<tr>
<th>Study</th>
<th>Fasting Time</th>
<th>Administration Procedure of D2O (99%)</th>
<th>Methods</th>
<th>Fractional Gluconeogenesis, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chacko et al. (6)</td>
<td>Overnight</td>
<td>1 g/kg × 5</td>
<td>Average</td>
<td>48</td>
</tr>
<tr>
<td>Landau et al. (16)</td>
<td>14 h</td>
<td>2.5 g/kg × 2 + 0.5% in drinking water</td>
<td>HMT</td>
<td>46</td>
</tr>
<tr>
<td></td>
<td>22 h</td>
<td></td>
<td>HMT</td>
<td>50</td>
</tr>
<tr>
<td>Hundal et al. (13)</td>
<td>15 h</td>
<td>1.25 g/kg × 4 + 0.5% in drinking water</td>
<td>HMT CS/C2</td>
<td>55</td>
</tr>
<tr>
<td>Chandramouli et al. (7)</td>
<td>14–16 h</td>
<td>3.5 ml/kg</td>
<td>HMT CS/C2 and 2H2O/C2</td>
<td>53</td>
</tr>
<tr>
<td>Allick et al. (1)</td>
<td>16 h</td>
<td>1 g/kg × 5 + 0.5% in drinking water</td>
<td>HMT CS/C2 and 2H2O/C2</td>
<td>45</td>
</tr>
<tr>
<td>Basu et al. (3)</td>
<td>Overnight</td>
<td>1.67 g/kg × 3</td>
<td>Average: CS/C2</td>
<td>72</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>and CS2H2O</td>
<td>68</td>
</tr>
<tr>
<td>Veldhorst et al. (30)</td>
<td>14 h</td>
<td>1 g/kg × 5 + 0.5% in drinking water</td>
<td>HMT: CS2H2O</td>
<td>64</td>
</tr>
<tr>
<td>Our study</td>
<td>14 h</td>
<td>1.67 g/kg × 3 + 0.5% in drinking water</td>
<td>Average: CS2H2O</td>
<td>68</td>
</tr>
<tr>
<td></td>
<td>22 h</td>
<td>(meat ingestion)</td>
<td></td>
<td>75</td>
</tr>
</tbody>
</table>

HMT, hexamethylenetetramine.
esis and glycogenolysis fluxes (Table 2) using the results of our previous study that was conducted in comparable conditions, i.e., after the ingestion of a single meal consisting of eggs supplying 23 g protein and 19 g fat after fasting overnight. The EGP we observed in the fasting state (10 ± 1.5 μmol·kg⁻¹·min⁻¹) was consistent with the literature (4, 14, 18, 26, 27, 30). Indeed, the pooled means ± SD from these studies is 10.9 ± 1.4 μmol·kg⁻¹·min⁻¹. Using these previous results, we simulated a mean glucosegenesis flux decreasing from 0.60 to 0.34 mg·kg⁻¹·min⁻¹ during the postprandial phase, and a gluconeogenesis flux ranging from 1 to 1.4 mg·kg⁻¹·min⁻¹, both ranges consistent with other studies (7, 12). Interestingly, the flux seems to increase within the 4 h following meat ingestion, a period that corresponds to the maximal delivery of dietary protein in the intestine (21). In the previous study (9), the flux of glucose produced from dietary amino acids was also determined, using an intrinsic ¹³C labeling of dietary protein. Applied to our data and considering the gluconeogenesis flux from dietary amino acids, this would have resulted in a contribution of dietary protein with a maximal value of 13.5% of gluconeogenesis during the postprandial ingestion with a maximum reached at 4 h. This extrapolation strongly supports previous observations that in the context of a high-protein low-carbohydrate diet, gluconeogenesis partially compensates for the decrease of glucose from glycogenolysis but not enough to maintain EGP (4). In consequence, the role of gluconeogenesis in the satiating effect of proteins remains unclear, as already questioned by the absence of any correlation between appetite ratings and increase of gluconeogenesis flux (31).

Fractional gluconeogenesis measured in this study appears to be 10% higher than in some other studies (1, 6, 7, 13, 16) but not all (3, 30), as shown in Table 3. Methodological differences may explain the variations of fractional gluconeogenesis, such as the administration procedure for oral doses of D₂O and analytical methods (average method vs. HMT derivative of glucose, and C₂ vs. plasma or urine body water ²H enrichment). The average method has been shown to be comparable to the HMT method, with positive differences of 1% to 5% (5, 6, 8). D₂O as a surrogate for ³H enrichment at C₂ was reported either to have no impact (1) or to produce lower values of gluconeogenesis than C₂ (16). Moreover, the doses of oral administration of the tracer varies among studies and can affect the isotope steady-state. On the day after oral administration, we gave subjects drinking water enriched at 0.5%, as specified in several studies. However, the steady-state is questionable because although variations were very low, we nevertheless observed a constant and significant decrease of ²H₂O over the postprandial period. In other studies, variations of 0.02%–0.03% MPE in C₂ or ³H₂O occurred within the observation period (1, 6). Such variation of deuterium enrichment in the body water pool would impact the fractional gluconeogenesis by 3%–4%. The fact that we performed five measurements every 2 h for 8 h reveals a very slight but systematic decrease. It is possible that the drinking water enriched at 0.5% was not sufficient to maintain the plateau in this study. Another point that may require further normalization is the composition of the last meal given the day before the experiment, which can strongly impact the glycogen store. In our study, a standard meal was given at 19:00, but the intake was not recorded. It is thus possible that the variation of fractional gluconeogenesis among subjects at t = 0 (57% to 78%) is due to differences in amount intake.

In conclusion, the present results show that after fasting overnight and following the ingestion of a single meat meal, the fractional gluconeogenesis progressively increased from 68% to 75%–79%. Using EGP obtained from a previous study that was conducted in similar conditions, we simulated a slight increase of gluconeogenesis flux during the 4 h following the protein meal ingestion.

ACKNOWLEDGMENTS

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DISCLOSURES

J. P. Godin and I. Breton are employed by Nestle; however, they have no conflicts of interest regarding this study. None of the other authors has any conflicts of interest, financial or otherwise, to disclose.

AUTHOR CONTRIBUTIONS


REFERENCES