Effect of protein restriction on 15N transfer from dietary [15N]alanine and [15N]Spirulina platensis into urea

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Hamadeh, Mazen J., and L. John Hoffer. Effect of protein restriction on 15N transfer from dietary [15N]alanine and [15N]Spirulina platensis into urea. Am J Physiol Endocrinol Metab 281: E349–E356, 2001.—Six normal men consumed a mixed test meal while adapted to high (1.5 g·kg−1·day−1) and low (0.3 g·kg−1·day−1) protein intakes. They completed this protocol twice: when the test meals included 3 mg/kg of [15N]alanine ([15N]Ala) and when they included 30 mg/kg of intrinsically labeled [15N]Spirulina platensis ([15N]SPI). Six subjects with insulin-dependent diabetes mellitus (IDDM) receiving conventional insulin therapy consumed the test meal with added [15N]Ala while adapted to their customary high-protein diet. Protein restriction increased serum alanine, glycine, glutamine, and methionine concentrations and reduced those of leucine. Whether the previous diet was high or low in protein, there was a similar increase in serum alanine, methionine, and branched-chain amino acid concentrations after the test meal and a similar pattern of 15N enrichment in serum amino acids for a given tracer. When [15N]Ala was included in the test meal, 15N appeared rapidly in serum alanine and glutamine, to a minor degree in leucine and isoleucine, and not at all in other circulating amino acids. With [15N]SPI, there was a slow appearance of the label in all serum amino acids analyzed. Despite the different serum amino acid labeling, protein restriction reduced the postmeal transfer of dietary 15N in [15N]Ala or [15N]SPI into [15N]urea by similar amounts (38 and 43%, respectively, not significant). The response of the subjects with IDDM was similar to that of the normal subjects. Information about adaptive reductions in dietary amino acid catabolism obtained by adding [15N]Ala to a test meal appears to be equivalent to that obtained using an intrinsically labeled protein tracer.

Dietary protein restriction induces an adaptive reduction in urea production over the hours after consumption of a constant-composition test meal (22). There is also reduced transfer of the 15N in a tracer dose of [15N]Ala included in the test meal into urea, suggesting that first-pass splanchnic amino acid retention is involved in the adaptive process (22). This reduction in 15N transfer into urea was less successful in protein-restricted persons with conventionally treated insulin-dependent diabetes mellitus (IDDM), raising the possibility that dietary protein requirements are increased for some persons with IDDM (21, 22).

However, tracer [15N]Ala is not protein bound, so its metabolism may not represent the extent to which the amino acids in dietary proteins are oxidized or conserved for protein synthesis upon their first passage through the splanchnic bed (29). We have, therefore, conducted a controlled trial of feeding subjects a mixed test meal containing either [15N]Ala or [15N]SPI, a 15N-labeled intact protein tracer (3), before and after protein restriction.

In earlier studies, we used a 0.5 g protein/kg test meal to examine the effect of previous diet on the efficiency of fed-state protein retention (22, 39). A test meal containing less protein ought to be a more sensitive tool, because optimal retention of the amino acids in such a meal calls for greater metabolic efficiency. The test meal in the present study contained 0.25 g protein/kg body wt. It was offered to normal research subjects before and after 3 days of adaptation to protein restriction. Each subject underwent the same protocol twice, the replicate protocols differing only in that a tracer dose of [15N]Ala was included in the test meals for three subjects the first time they followed the protocol and a tracer dose of fully 15N-labeled whole protein, [15N]SPI, the second time, with the order reversed for the other three subjects. Measurements were also made in healthy persons with IDDM receiving conventional insulin therapy while adapted to their customary high-protein intake, and their results were compared with those of the normal subjects.

The goals of this study were 1) to test whether the reduction in the transfer of 15N added to a meal as [15N]Ala into urea after protein restriction is comparable to what occurs after ingestion of [15N]SPI, an intrinsically labeled protein; and 2) to compare the distribution of the different 15N-labeling vectors in serum amino acids to gain insight into the validity of using the fate of [15N]Ala as a marker for dietary free amino N in future studies.

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METHODS

Subjects and protocols. Six healthy, nonsmoking men using no medications and with normal blood chemistries were admitted at 0700 to the clinical research unit, where they consumed a diet providing 38 kcal/kg and 1.5 g protein/kg on that day. The following morning (day 2) was the first test meal study, after which the subjects followed a low-protein diet for the rest of the day for total energy and protein intakes of 38 kcal/kg and 0.39 g/kg, respectively. Protein restriction (0.31 g/kg, with maintenance energy) continued on days 3 and 4. The test meal was repeated on the morning of day 5. The protocol was repeated 10 days later, the replicate protocols differing only in that a tracer dose of [15N]Ala was included in the test meals for three subjects on the first occasion, and a tracer dose of fully [15N]-labeled whole protein, [15N]SPI, on the second one. The protocol order was reversed for the other three subjects. Four healthy men and two healthy women with IDDM presented to the research unit at 0700 for a study that they completed the same day. Their blood hemoglobin A1c concentrations were 6.20700 for a study that they completed the same day. Their healthy women with IDDM presented to the research unit at 0700 for a study that they completed the same day. Their study was approved by the Research and Ethics Committee of the Jewish General Hospital in Montreal.

Test meal. The test meal was Glucerna (Ross Laboratories, St. Laurent, QC, Canada) to which beet sugar (Rogers Sugar, Winnipeg, Manitoba, Canada) was added to provide 0.25 g of protein and 10 kcal of energy/kg body wt (10% protein, 30% fat, and 60% carbohydrate). Given together with the test meal was either 3 mg [15N]Ala/kg body wt (99%; MSD Isotopes, Montreal, QC, Canada) or 30 mg of processed dried [15N]SPI/kg body wt (99% 15N; Martek, Columbia, MD). To each meal was also added 200 mg of [15C]urea (99%; MSD Isotopes) with the aim of using the difference between the amount of tracer administered and the amount of it found in the serum and urine after the meal as a measure of extraneous urea losses.

Intrinsically 15N-labeled Spirulina platensis was purchased from Martek Biosciences and confirmed to be 99% 15N by isotope ratio-mass spectrometry (IRMS) on a Kjeldahl digestate. One gram of untreated algae contained 320 mg of glycogen (26). The following procedure was developed to remove nonprotein N (free nucleotides, free amino acids, and nucleic acids) (24). Perchloric acid (30–35 ml of 0.5 N in saline) was added to each 3-g batch of the dry product, thoroughly mixed, and incubated in a 70°C water bath for 20 min. The mixture was centrifuged at 200 g for 10 min at room temperature. The acid supernatant was decanted, and the procedure was repeated. After the addition of 40–45 ml of ethanol, the mixture was centrifuged at 200 g for 10 min at room temperature. The ethanol was decanted, and the procedure was repeated five times to remove the perchlorate and chlorophyll, after which the resulting product was oven dried at 60°C. To determine protein N, 30 mg of the dried algal protein residue and 10 mg of catalyst (9 g K2SO4; 1 g CuSO4) were digested with 1 ml of acid mixture (7 g SeO2 in 1 liter of concentrated H2SO4 and 1 ml of H2O at 125°C for 30 min, then at 250°C for 17 min, and then at 450°C for 45 min (30). The volume was brought to 40 ml with a solution of 100 mmol/l sodium phosphate (pH 5.8), and 1 ml was diluted with 0.8 ml of 1 mol/l NaOH and 0.2 ml of H2O and measured on an Antek 7000 elemental analyzer (Antek Insustems, Houston, TX). A typical lot of dried algal protein residue contained 98–118 mg N/g body wt. The administered 15N dose per kilogram of body weight was 0.470 ± 0.002 mg/kg for

![Fig. 1. Serum alanine enrichment and concentration before ([15N]alanine with high-protein ([15N]Ala-HP), ■; [15N]Spirulina platensis ([15N]SPI)-HP, ●); and after ([15N]Ala with low-protein (LP), □; [15N]SPI-LP, ○) protein restriction. TTR, tracer-to-tracee ratio. Serum concentrations were significantly higher on the LP diet. Different letters denote significantly different values. *Significantly different from postabsorptive concentration (P < 0.05).](http://ajpendo.physiology.org/)
[15N]Ala and 3.09 ± 0.08 mg/kg for [15N]SPI, of which ~0.55 mg was in alanine (3).

Analytical methods. Serum amino acid concentrations and enrichments were analyzed by gas chromatography-mass spectrometry (GC-MS), as previously described (19). To 100 μl of serum were added the internal standards, 2.5 μg of norleucine (Sigma Chemical, St. Louis, MO), 2.5 μg of L-[3,3,3-2H3]alanine (99.4% 2H, MSD Isotopes), 2 μg of [2,2-2H2]glycine (CDN Isotopes, Pointe-Claire, QC, Canada), 0.5 μg of L-[S-methyl-2H3]methionine (CDN Isotopes), 0.1 μg of L-[3,4-13C2]aspartate (MassTrace, Woburn, MA), and 10 μg of L-[3,3,4,4-2H4]glutamine (Tracer Technologies, Somerville, MA), and, after acidification with 1.5 ml of 1 mol/l acetic acid, the sample was applied to 1-ml columns of cation exchange resin (Dowex 50W-X8, 100–200 mesh, hydrogen form, Bio-Rad Laboratories, Richmond, CA). The amino acids were eluted into 3.7-ml flat-bottomed vials equipped with Teflon-lined caps (Du Pont de Nemours, Wilmington, DE) with four sequential 1-ml additions of 3 mol/l NH4OH. The NH4OH fraction was evaporated under a gentle stream of N2. Tert-butyldimethylsilyl (TBDMS) derivatives were prepared as described by Patterson et al. (33).

GC-MS analyses were performed using an HP-5890 gas chromatograph (Hewlett-Packard, Palo Alto, CA) directly...
coupled to an HP-5988A quadrupole mass spectrometer. Samples were introduced by splitless injection (1.0 μl) from an HP-7673 autoinjector onto a fused silica DB-1 capillary column (30 × 0.25 mm, 0.25-μm film thickness, J&W Scientific, Folsom, CA) under the following GC conditions: initial column temperature, 110°C (maintained for 2 min); program rate, 6°C/min until 200°C and then 10°C/min to a final column temperature of 200°C; helium carrier gas column head pressure, 70 kPa; and injector port and transfer line temperatures, 250°C. The electron impact mass spectrometry conditions were as follows: ionizing energy, 70 eV; emission current, 300 μA; and source temperature, 200°C. The electron impact mass spectrometry conditions were as follows: ionizing energy, 70 eV; emission current, 300 μA; and source temperature, 200°C. The following ions were monitored by selected ion monitoring: TBDMS-alanine [mass-to-charge ratio (m/z) 158.1], TBDMS-[15N]alanine (m/z 159.1), TBDMS-[3,3,3-2H3]alanine (m/z 161.1), TBDMS-glycine (m/z 218.1), TBDMS-[15N]glycine (m/z 219.1), TBDMS-[2,2,2-3H3]glycine (m/z 220.1), TBDMS-leucine (m/z 200.2), TBDMS-[15N]leucine (m/z 201.2), TBDMS-isoleucine (m/z 200.2), TBDMS-[15N]isoleucine (m/z 201.2), TBDMS-norleucine (m/z 200.2), TBDMS-methionine (m/z 218.1), TBDMS-[15N]methionine (m/z 219.1), TBDMS-[S-methyl-2H3]methionine (m/z 221.1), TBDMS-serine (m/z 390.3), TBDMS-[15N]serine (m/z 391.3), TBDMS-aspartate (m/z 418.2), TBDMS-[15N]aspartate (m/z 419.2), TBDMS-[3,4-13C2]aspartate (m/z 420.2), TBDMS-glutamine (m/z 432.3), TBDMS-[15N]glutamine (m/z 433.3), and TBDMS-[3,3,4,4-2H4]glutamine (m/z 436.3).

Fig. 4. Serum isoleucine enrichment and concentration before ([15N]Ala-HP, ■; [15N]SPI-HP, ●) and after ([15N]Ala-LP, □; [15N]SPI-LP, ○) protein restriction. Different letters denote significantly different values. *Significantly different from postabsorptive concentration (P < 0.05).

Fig. 5. Serum methionine enrichment and concentration before ([15N]Ala-HP, ■; [15N]SPI-HP, ●) and after ([15N]Ala-LP, □; [15N]SPI-LP, ○) protein restriction. Serum concentrations were significantly higher on the LP diet. Different letters denote significantly different values. *Significantly different from postabsorptive concentration (P < 0.05).
Serum and urinary [15N]urea enrichments were determined by Metabolic Solutions (Merrimack, NH) by means of a Europa Tracer-Mass IRMS (Europa Scientific, Crewe, UK), with the N2 generated from ammonium sulfate used as the reference gas, according to the method of Read et al. (34). This method converts urea N into NH3, eliminating any contribution from 13C and 18O. 15N recovery in urea was calculated as [15N]urea excretion over the 9 h after the test meal plus the amount of [15N]urea present in total body water (TBW) at hour 9 divided by 15N intake.

Statistical analysis. Three-way repeated-measures ANOVA was used to determine significant differences between data for the normal group. The three factors were diet (high vs. low protein), 15N source ([15N]Ala vs. [15N]SPI), and time for serum amino acid and urea concentrations. Two-way repeated-measures ANOVA was used to determine significant differences in 15N enrichment and recovery, serum postabsorptive and 2-h postmeal amino acid and urea concentrations, with the two factors being diet and 15N source. For urea and amino acid enrichments, the two factors were diet and time within the same 15N source. Within the same 15N source and protein level, serum urea concentration over time was subjected to one-way repeated-measures ANOVA. When significance occurred, a Newman-Keuls test was used post hoc to determine the source of difference. Student’s unpaired t-test and two-way repeated-measures ANOVA were used to determine significant differences between the normal and IDDM groups, also using the Newman-Keuls test to determine the
source of difference. Differences were considered significant at P ≤ 0.05. Results are presented as means ± SE unless otherwise indicated.

RESULTS

Three days of protein restriction increased the serum concentrations of alanine (P = 0.0001), glycine (P = 0.0001), glutamine (P = 0.0001), and methionine (P = 0.0001), while decreasing those of leucine (P < 0.002) (Figs. 1–7). Whether or not the previous diet was high or low in protein, the test meal stimulated generally similar increases in serum alanine, leucine, isoleucine, and methionine and no change in serum glycine, glutamine, or aspartate concentrations (Figs. 1–7). The amino acid labeling pattern characteristic of each 15N tracer was also generally unaffected by whether the previous diet was high or low in protein (Figs. 1–7).

By contrast, the form of 15N-labeled amino acid tracer used with the test meal greatly affected serum amino acid labeling. After [15N]Ala, label appeared rapidly in serum alanine and glutamine, to a minor degree in leucine and isoleucine, and not at all in other rapidly in serum alanine and glutamine, to a minor degree in leucine and isoleucine, and not at all in other circulating amino acids. After [15N]SPI, there was a slow appearance of the label in all serum amino acids analyzed, with no preference for alanine or glutamine.

As measured 9 h after the test meal, protein restriction reduced the transfer of 15N into urea by 38% with the use of [15N]Ala and by 43% with the use of [15N]SPI. This difference was not statistically significant.

The IDDM subjects consumed the test meal with [15N]Ala while adapted to a conventional, high-protein diet. Their serum urea and amino acid concentrations were close to normal, except that postabsorptive serum alanine and glutamine concentrations were higher than normal (Table 1). Postmeal 15N-labeled amino acid enrichments (including those in alanine) were similar to those of the normal subjects (data not shown). Their 15N transfer into urea was normal (Table 2).

DISCUSSION

The main conclusion of this study is that addition of [15N]Ala to a test meal provided information about an adaptive change in fed-state amino acid catabolism equivalent to what was obtained using an intrinsically labeled protein tracer. This was obtained despite very different serum amino acid 15N labeling and different absolute recoveries of the administered 15N dose in urea.

A trace amount of [13C]urea was included in the test meals to verify the accuracy of the [15N]urea recovery results. Measurement of [15N]urea production involves adding the amount of the tracer in urine collected over the 9-h postmeal observation period to that in TBW at the final time point, as described by Fern et al. (12) in their single-dose [15N]glycine end-product model for whole body N turnover. The calculation ignores any loss of urea (and urea tracer) in the gut or elsewhere (16, 27, 38, 44, 46). It also ignores the possibility that diet or disease could change renal urea clearance, which in turn could introduce systematic error by changing the distribution of urea between TBW and urine (17). Our finding that [13C]urea recovery was unaffected by diet or IDDM allays concern that these may have had a distorting effect on [15N]urea recovery. As is noted in the companion article (20), the [13C]urea recoveries were higher than anticipated, perhaps due to an imprecision in our mathematical correction for

Table 1. Postabsorptive and 2-h postmeal serum urea and amino acid concentrations

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Normal</th>
<th></th>
<th>IDDM</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Postabsorptive</td>
<td>Postmeal</td>
<td>Postabsorptive</td>
<td>Postmeal</td>
</tr>
<tr>
<td>Urea, mmol/l</td>
<td>4.4 ± 0.4</td>
<td>4.2 ± 0.4</td>
<td>4.1 ± 0.7</td>
<td>3.9 ± 0.7</td>
</tr>
<tr>
<td>Leucine, µmol/l</td>
<td>138 ± 7</td>
<td>166 ± 12</td>
<td>97 ± 9†</td>
<td>142 ± 12‡</td>
</tr>
<tr>
<td>Isoleucine, µmol/l</td>
<td>68 ± 3</td>
<td>90 ± 6§</td>
<td>54 ± 6†</td>
<td>78 ± 7‡</td>
</tr>
<tr>
<td>Methionine, µmol/l</td>
<td>25 ± 2</td>
<td>36 ± 2§</td>
<td>19 ± 1†</td>
<td>32 ± 3‡</td>
</tr>
<tr>
<td>Alanine, µmol/l</td>
<td>300 ± 21</td>
<td>544 ± 17ª</td>
<td>331 ± 53</td>
<td>538 ± 49ª</td>
</tr>
<tr>
<td>Glutamine, µmol/l</td>
<td>454 ± 37</td>
<td>518 ± 41ª</td>
<td>562 ± 31†</td>
<td>653 ± 31†</td>
</tr>
<tr>
<td>Glycine, µmol/l</td>
<td>228 ± 13</td>
<td>227 ± 17</td>
<td>278 ± 27</td>
<td>308 ± 32</td>
</tr>
<tr>
<td>Aspartate, µmol/l</td>
<td>18 ± 4</td>
<td>16 ± 2</td>
<td>13 ± 2</td>
<td>12 ± 2</td>
</tr>
</tbody>
</table>

Data are presented as means ± SE. IDDM, insulin-dependent diabetes mellitus. *Significantly different from postabsorptive (P < 0.05); †significantly different from normal (P < 0.05).

Table 2. Postmeal 15N transfer into urea

<table>
<thead>
<tr>
<th>Previous protein intake</th>
<th>[15N]Ala</th>
<th></th>
<th>[15N]SPI</th>
<th></th>
<th>[15N]Ala</th>
</tr>
</thead>
<tbody>
<tr>
<td>High</td>
<td>0.475 ± 0.041</td>
<td>0.296 ± 0.045</td>
<td>0.978 ± 0.122</td>
<td>0.542 ± 0.104</td>
<td>0.330 ± 0.053</td>
</tr>
<tr>
<td>Low</td>
<td>20.1 ± 1.4</td>
<td>12.5 ± 1.8ª</td>
<td>6.3 ± 1.0†</td>
<td>3.6 ± 0.8†</td>
<td>15.6 ± 5.0</td>
</tr>
</tbody>
</table>

Data are presented as means ± SE. [15N]Ala, [15N]alanine; [15N]SPI, [15N]Spirulina platensis. *Significantly different from the low protein diet (P < 0.05); †significantly different from [15N]Ala (P < 0.05).
the contribution of \([^{15}N]urea\) to the \([M+1]urea\) mass. Although it is possible that \([^{15}N]urea\) synthesis partially distorted our \([^{13}C]urea\) recovery measurement, the reverse could not occur, because the IRMS analysis converts urea to ammonia, eliminating any contribution from \(^{13}C\) or \(^{18}O\).

Figures 1–7 illustrate that serum amino acid concentration profiles were closely similar when the dietary protocol was repeated and that the postmeal serum amino acid \([^{15}N]\)-labeling pattern was similar for a given \(^{15}N\) tracer whether the preceding diet was high or low in protein. However, the labeling patterns were very different with the different tracers. After \([^{15}N]Ala\), the \(^{15}N\) tracer can be assumed to have been rapidly absorbed and a large amount of it deaminated (2, 25, 45). After \([^{15}N]SPI\), \(^{15}N\)-labeled amino acids were released into the circulation slowly. There was greater total \(^{15}N\)urea production after \([^{15}N]SPI\); this can be attributed to its larger \(^{15}N\) dose. The greater recovery of \(^{15}N\) from \([^{15}N]Ala\) in urea is a consequence of its rapid deamination compared with other amino acids (2, 25, 45). The same phenomenon is observed when whole body \(N\) flux is measured using the single-dose end-product model, which calculates flux as the \(^{15}N\) dose divided by the \(^{15}N\) enrichment in urea produced over the subsequent 12 h (12). The flux obtained when the dose is administered as an intrinsically labeled protein is approximately twice that obtained using \([^{15}N]Ala\) (13). We presume that very rapid mixing of \(^{15}N\) from \([^{15}N]Ala\) in the urea precursor pool is the reason why our main outcome measure, fractional change in tracer appearance in urea, was closely similar for \([^{10}N]Ala\) and \([^{15}N]SPI\).

The notion that splanchnic first-pass amino acid conservation or catabolism is important in nutritional adaptation is supported by several studies that have used sophisticated tracer approaches in humans and piglets (4, 8, 23, 29, 35, 36). Notwithstanding the complex nature of splanchnic amino acid metabolism indicated by these studies and the important effects on model parameters when individual tracer amino acids or intrinsically labeled protein tracers are used (6, 29), it may be useful, in certain situations, to bypass the details of how the body achieves its homeostatic aims and, as in the present study, simply measure the system’s regulated output: short-term amino acid catabolism after a metabolic or nutritional challenge.

The present results support such a conceptual approach. They suggest that, despite serum amino acid \(^{15}N\) labeling that differed greatly from what was produced by an intrinsically labeled protein, \([^{15}N]Ala\) gave a similar system output, i.e., an equivalent reduction in \(^{15}N\) tracer incorporation into urea. The advantages of this method are simplicity and robustness. These render it amenable to use in a variety of clinical settings, with the potential for insights that can be coupled with the results of more sophisticated tracer methodologies.

We found no indication of excess postmeal transfer of \(^{15}N\) from \([^{15}N]Ala\) into urea in persons with mildly hyperglycemic IDDM. This observation is consistent with their normal postmeal sulfate and total urea production (20) and with our earlier finding in conventionally treated IDDM by using a high-protein test meal (22). Insulin withdrawal increases urea production (1, 14) and leucine plasma concentration, oxidation, and turnover in IDDM (31, 32). Conventional IDDM therapy is also commonly associated with increased circulating branched-chain amino acid concentrations (11, 28, 37, 41), but, as in the present study, this is not always the case (7, 42, 43). Where and how does insulin therapy of IDDM regulate amino acid incorporation into body proteins? At one level, it restrains muscle proteolysis, a restraint that is released when insulin provision is grossly inadequate (31, 32). Tracer studies of postmeal (40) or fed-state (5) amino acid kinetics in insulin-deprived IDDM indicate defective suppression of whole body proteolysis before the meal, with persistence to a varying extent into the fed state. Milder insulin-deficient states may yet suffice to restrain proteolysis, while exerting anabolic and regulatory effects when injected insulin, together with dietary amino acids, reaches the liver (10) and the periphery to stimulate muscle protein synthesis (15). We suggest that the low serum leucine concentrations measured in our IDDM subjects are evidence that they had sufficient exogenous insulin in their tissues to prevent a protein-catabolic state despite their hyperglycemia. The results would presumably have been different had their insulin deficiency been more severe.

In conclusion, we have found that inclusion of \([^{15}N]Ala\) in a test meal provided information about adaptive changes in dietary amino acid catabolism equivalent to that provided by the use of an intrinsically labeled protein tracer. Under conditions of adaptation to a high protein intake, persons with conventionally treated IDDM demonstrated normal first-pass dietary amino acid retention.

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