Measurement of human mixed muscle protein fractional synthesis rate depends on the choice of amino acid tracer

Gordon I. Smith, Dennis T. Villareal, and Bettina Mittendorfer

Washington University School of Medicine, St. Louis, Missouri

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Smith GI, Villareal DT, Mittendorfer B. Measurement of human mixed muscle protein fractional synthesis rate depends on the choice of amino acid tracer. Am J Physiol Endocrinol Metab 293: E666–E671, 2007. First published May 29, 2007; doi:10.1152/ajpendo.00185.2007.—The goal of this study was to discover whether using different tracers affects the measured rate of muscle protein synthesis in human muscle. We therefore measured the mixed muscle protein fractional synthesis rate (FSR) in the quadriceps of older adults during basal, postabsorptive conditions and mixed meal feeding (70 mg protein·kg fat-free mass−1·h−1 × 2.5 h) by simultaneous intravenous infusions of [5,5,5-2H3]leucine and either [ring-13C6]phenylalanine or [ring-2H5]phenylalanine and analysis of muscle tissue samples by gas chromatography-mass spectrometry. Both the basal FSR and the FSR during feeding were ~20% greater (P < 0.001) when calculated from the leucine labeling in muscle tissue fluid and proteins (fasted: 0.063 ± 0.005%/h; fed: 0.080 ± 0.007%/h) than when calculated from the phenylalanine enrichment data (0.051 ± 0.004 and 0.066 ± 0.005%/h, respectively). The feeding-induced increase in the FSR (~20%; P = 0.011) was not different with leucine and phenylalanine tracers (P = 0.69). Furthermore, the difference between the leucine- and phenylalanine-derived FSRs was independent of the phenylalanine isotopomer used (P = 0.92). We conclude that when using stable isotope-labeled tracers and the classic precursor product model to measure the rate of muscle protein synthesis, absolute rates of muscle protein FSR differ significantly depending on the tracer amino acid used; however, the anabolic response to feeding is independent of the tracer used. Thus, different precursor amino acid tracers cannot be used interchangeably for the evaluation of muscle protein synthesis, and data from studies using different tracer amino acids can be compared qualitatively but not quantitatively.

MUSCLE MASS IS MAINTAINED by a balance between the rate of muscle protein synthesis and breakdown (29), which can be measured in vivo by using a variety of tracer methods (4, 27, 28, 32, 40, 42). The most commonly used approach to measure the rate of muscle protein synthesis is based on the incorporation of a stable isotope-labeled amino acid (usually phenylalanine or leucine) during continuous intravenous infusion of the tracer to determine the muscle protein fractional synthesis rate (FSR) (5, 22, 31, 35, 37, 41). The reported rates of mixed muscle protein synthesis from these studies vary substantially even during standard overnight fasted conditions in young, healthy individuals (from ~0.04 to 0.08%/h) (11–13, 16, 23, 24, 36, 37, 41). The reason(s) for this variability in results is not clear.

One potential explanation for the disparity in results may be the use of different tracers and/or surrogate precursor pools for the calculation of the FSR. Although the FSR is known to be influenced, to some degree, by the precursor pool used for calculating the FSR (6–8, 18, 38), theoretically the FSR should be the same regardless of which labeled amino acid is infused (39). However, substantial differences in the measured muscle protein synthesis rate have been observed when different radioactively labeled amino acid tracers ([14C]leucine vs. [13C]phenylalanine) are used in rats (20). This casts doubt on this important assumption but could be due to errors in deriving the exact amino acid composition of muscle proteins, which is necessary when using radioactive amino acid tracers to measure muscle protein turnover. Studies in which the flooding dose (21) or limb arteriovenous balance (3, 33) techniques were used to examine this issue in human muscle also suggest that there may be an amino acid tracer dependence of the measured muscle protein synthesis rate. However, the results are difficult to interpret because of the small number of subjects included in the flooding dose study (21) and potential errors in measuring limb leucine oxidation or the leucine and phenylalanine content in muscle proteins with the AV balance technique (3, 33). Whether the measured rate of human muscle protein synthesis is tracer dependent when a continuous intravenous infusion of stable isotope-labeled tracers is used with simultaneous measurement of tracer incorporation into muscle proteins has never been investigated.

The purpose of the present study, therefore, was to evaluate how the choice of stable isotope-labeled tracer affects the calculated rate of human skeletal muscle protein synthesis. To do so, we measured the muscle protein FSR in the quadriceps of older men and women by simultaneous continuous intravenous infusion of [5,5,5-2H3]leucine and either [ring-2H5]phenylalanine or [ring-13C6]phenylalanine during basal, postabsorptive conditions and feeding.

METHODS

Subjects. Fifteen older adults (6 men and 9 women; age: 70 ± 1 yr; body mass index: 38.0 ± 1.6 kg/m²; means ± SE) who underwent measurements of their muscle protein FSR as part of a larger, currently ongoing clinical trial participated in this study. All subjects were considered to be in good health after completion of a comprehensive medical evaluation. Written informed consent was obtained from all subjects before their participation in the study, which was approved by the Human Studies Committee and the General Clinical Research Center (GCRC) Advisory Committee at Washington University School of Medicine (St. Louis, MO).

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Experimental protocol. Of the 15 subjects, 5 underwent one study, whereas the other 10 subjects underwent 2 separate studies (~3 mo apart), to determine the FSR of muscle proteins by simultaneous intravenous infusion of stable isotope-labeled leucine and phenylalanine tracers and analysis of blood and muscle tissue samples by mass spectrometry. The repeat studies in 10 of the subjects were performed after various types of “intervention” (e.g., exercise training, weight loss) and are therefore not reproducibility studies; hence, data from these studies were treated as independent observations. Subjects were admitted to the GCRC the evening before the tracer infusion study. At 2000, they consumed a standard meal, containing 12 kcal/kg body weight (55% of total energy from carbohydrates, 30% from fat, and 15% from protein) and then rested in bed until completion of the study the next day. At ~0600 on the following morning, a cannula was inserted into an antecubital vein for infusion of the stable isotope-labeled amino acids; another cannula was inserted into a vein of the contralateral forearm for blood sampling. At ~0800, a baseline blood sample was obtained to determine the background enrichments of leucine and phenylalanine in plasma, and a muscle biopsy was taken from the quadriceps femoris to determine the background leucine and phenylalanine enrichments in muscle protein and muscle tissue fluid. Immediately afterward, primed constant infusions of [5,5,5-2H3]leucine (priming: 4.8 μmol/kg body weight; infusion rate: 0.08 μmol·kg body weight⁻¹·min⁻¹) and either [ring-13C6]phenylalanine or [ring-2H5]phenylalanine (priming: 5.4 μmol/kg body weight; infusion rate: 0.05 μmol·kg body weight⁻¹·min⁻¹) were started and maintained until completion of the study some 6 h later. [Ring-13C6]phenylalanine was used in 14, and [ring-2H5]phenylalanine was used in 11, of the 25 studies. At 210 min after the start of the tracer infusion, an additional muscle biopsy was obtained to determine the basal rate of muscle protein synthesis (as incorporation of labeled amino acids into proteins; see Calculations). Immediately following the biopsy procedure, a liquid meal (Ensure; Abbott Laboratories, Abbott Park, IL) containing 15% of total energy as protein, 55% as carbohydrate, and 30% as fat was given in small boluses every 10 min for 150 min so that every person received a total of 70 mg protein/kg fat-free mass (FFM) per hour plus a priming dose of 23 mg protein/kg FFM during the 2.5-h feeding period. At the onset of the feeding regimen, the infusion rates of labeled leucine and phenylalanine were increased to 0.12 μmol·kg body weight⁻¹·min⁻¹ and 0.08 μmol·kg body weight⁻¹·min⁻¹, respectively, to adjust for the increased amino acid availability. A muscle biopsy was obtained again 150 min after ingestion of the first aliquot of the meal to determine the rate of muscle protein synthesis during mixed meal feeding. The second and third biopsies were obtained through the same incision on the leg, with the forceps directed in proximal and distal direction, respectively, so that the two biopsies were collected from sites that were ~10–20 cm apart; the first biopsy was taken from the opposite leg. Additional blood samples were obtained every 30 min during the entire experiment to determine plasma leucine and phenylalanine enrichment. The tracer infusions were stopped and cannulas removed after the last (third) biopsy and the final blood draw were completed.

Sample collection and storage. Blood samples (~3 ml) were collected in prechilled tubes containing EDTA; plasma was separated by centrifugation within 30 min of collection and then stored at ~80°C until final analyses were performed. Muscle tissue (~100 mg) was obtained under local anesthesia (lidocaine, 2%) by using a Tilley-Henkel forceps. The tissue was immediately frozen in liquid nitrogen and then stored at ~80°C for determination of leucine and phenylalanine enrichments in muscle protein and tissue fluid.

Sample processing and analyses. For determination of plasma leucine and phenylalanine enrichments (tracer-to-tracere ratios, TTR), proteins were precipitated and the supernatant, containing free amino acids, was collected to prepare their t-butyldimethylsilyl (t-BDMS) derivatives for analysis by gas chromatography–mass spectrometry (GC-MS; MSD 5973 System; Hewlett-Packard) via electron ionization and selective ion monitoring (26). For determination of leucine and phenylalanine enrichments in muscle proteins and muscle tissue fluid, muscle samples (~20 mg) were homogenized in 1 ml of trichloroacetic acid solution (3% wt/vol), proteins were precipitated by centrifugation, and the supernatant, containing free amino acids, was collected. The pellet containing muscle proteins was washed and then hydrolyzed in 6 N HCl at 110°C for 24 h. Amino acids in the protein hydrolysate and supernatant samples were then purified on cation-exchange columns (Dowex 50W-X8; 200–Bio-Rad Laboratories, Richmond, CA), and leucine and phenylalanine were converted to their t-BDMS derivatives to determine their respective TTRs by GC-MS analysis (MSD 5973 System; Hewlett-Packard) with [5,5,5-2H3]leucine, [ring-2H5]phenylalanine, and [ring-13C6]phenylalanine enrichment standards and selective ion monitoring after electron ionization (26). The following mass-to-charge ratios were monitored in the muscle protein extracts: 202 (m+2) and 203 (m+3) for [5,5,5-2H3]leucine, 237 (m+3) and 239 (m+5) for [ring-2H5]phenylalanine, and 238 (m+4) and 240 (m+6) for [ring-13C6]phenylalanine.

Calculations. The FSR of mixed muscle protein was calculated on the basis of the incorporation rate of [5,5,5-2H3]leucine, [ring-2H5]phenylalanine, or [ring-13C6]phenylalanine into muscle proteins by using a standard precursor-product model as follows: FSR = \( \frac{E_p}{E_c} \times \frac{1}{100} \), where \( E_c \) is the mean enrichment over time of the precursor for protein synthesis, and \( E_p \) the time the enrichment between the precursors. The free leucine or phenylalanine enrichment in muscle tissue fluid was chosen to represent the immediate precursor for muscle protein synthesis (i.e., aminoacyl-t-RNA) (38). Values for FSR are expressed as percent per hour.

Statistical analysis. All data sets were tested for normality, and the differences in FSR derived from the leucine and phenylalanine tracer enrichments were evaluated using either repeated-measures analysis of variance with tracer and study condition (fasted vs. fed) as the factors or paired Student’s t-test, as appropriate. A P value ≤0.05 was considered statistically significant.

RESULTS

Leucine and phenylalanine concentrations in plasma and enrichments in plasma, muscle tissue fluid, and muscle proteins. Fasting plasma free leucine concentrations was 121 ± 5 μM and fasting plasma phenylalanine concentration was 71 ± 3 μM; both increased by ~15% during feeding to 134 ± 6 and 81 ± 3 μM, respectively. Plasma free leucine and phenylalanine enrichments were constant during both basal, postabsorptive conditions and feeding (data not shown), but the enrichment was ~35% greater during feeding than postabsorptively (Table 1). Muscle free leucine and phenylalanine enrichments were ~45% greater during feeding compared with postabsorptive conditions (Table 1). Muscle proteins were significantly enriched above baseline levels with the leucine and phenylalanine tracers (~0.011%) at the end of the basal, postabsorptive period; the enrichment increased further (to ~0.028%) during feeding until the end of the study, 2.5 h later (Table 1).

Muscle protein synthesis. The FSR calculated from the leucine labeling in muscle tissue fluid and proteins was ~20% greater (\( P < 0.001 \)), during both basal, postabsorptive conditions and feeding, than the respective FSRs calculated from the phenylalanine enrichment data (Fig. 1). However, the feeding induced increase in the FSR (\( P = 0.011 \)) was not different with the two tracers (Fig. 2). The discrepancy between the leucine- and phenylalanine-derived FSRs was independent of the phenylalanine isotopomer used; the differences between the leucine-derived FSR and the FSR derived from
provide similar values for the human muscle protein synthesis during basal, postabsorptive conditions and during feeding. [5,5,5-\(^2\)H\(_3\)]leucine was used in all 25 studies; [ring-\(^2\)H\(_5\)]phenylalanine was used in 11 studies; [ring-\(^1\)C\(_6\)]phenylalanine was used in 14 studies. In plasma, values are averages during basal, postabsorptive and fed conditions. In muscle tissue fluid and proteins, values were obtained at the end of the basal period (i.e., 210 min after the start of the tracer infusion) and the end of the feeding period (i.e., 360 min after the start of the tracer infusion).

**DISCUSSION**

It was our goal to discover whether or not primed, constant intravenous infusions of different stable isotope-labeled amino acid tracers with GC-MS analysis of muscle tissue samples provide similar values for the human muscle protein synthesis rate during basal, postabsorptive conditions and during feeding. We therefore measured the mixed muscle protein FSR simultaneously with a [\(^5\)H\(_5\)]leucine and either a [ring-\(^2\)H\(_5\)]phenylalanine or [ring-\(^1\)C\(_6\)]phenylalanine tracer. We found that the FSR derived from leucine labeling in muscle tissue fluid and proteins was ~20% greater than the FSR derived from phenylalanine labeling in muscle tissue fluid and proteins, during both fasted and fed conditions. The difference in results was independent of the phenylalanine tracer labeling (i.e., \(^2\)H\(_5\) vs. \(^1\)C\(_6\)). Furthermore, the anabolic response to feeding was also independent of the tracer used. Thus qualitative comparison of the results from studies in which different tracer amino acids were used is valid, but quantitative comparison is not.

Both the phenylalanine and leucine tracer infusions in conjunction with muscle tissue analysis by GC-MS provided equally robust measurements of the muscle protein FSR. The population variance in our study was ~35–40% independent of the tracer used. This is generally comparable to reports in the literature (11, 16, 17, 24, 31, 34), in particular one in which the same techniques were used in a similar subject population (37). However, we (22) and others (10, 13, 25) have previously obtained much tighter values in healthy, young men when using GC-combustion isotope ratio mass spectrometry (GC-C-IRMS) for the analysis of muscle protein samples, probably because of the greater sensitivity of GC-C-IRMS compared with GC-MS analysis (39).

The absolute mixed muscle protein FSR values obtained in our present study are also in good agreement with the values

<table>
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<th>Tracer</th>
<th>Fasted</th>
<th>Fed</th>
<th>P-value</th>
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<tbody>
<tr>
<td>[5,5,5-(^2)H(_3)]leucine</td>
<td>0.0708 ± 0.0027</td>
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Values (tracer-to-tracee ratios corrected for the background enrichment) are means ± SE and represent leucine and phenylalanine enrichments in plasma, muscle tissue fluid, and muscle proteins during basal, postabsorptive conditions (fasted) and feeding. [5,5,5-\(^2\)H\(_3\)]leucine was used in all 25 studies; [ring-\(^2\)H\(_5\)]phenylalanine was used in 11 studies; [ring-\(^1\)C\(_6\)]phenylalanine was used in 14 studies. In plasma, values are averages during basal, postabsorptive and fed conditions. In muscle tissue fluid and proteins, values were obtained at the end of the basal period (i.e., 210 min after the start of the tracer infusion) and the end of the feeding period (i.e., 360 min after the start of the tracer infusion).

**Fig. 1.** Skeletal muscle protein fractional synthesis rate (FSR) during basal, postabsorptive conditions (fasted) and feeding (fed) measured using stable isotope-labeled leucine and phenylalanine tracers. Values are means ± SE. ANOVA revealed significant effects for tracer amino acid used (P < 0.001) and feeding (P = 0.011) but no interaction (P = 0.80).

**Fig. 2.** Feeding-induced increase in skeletal muscle protein FSR with the use of stable isotope-labeled leucine and phenylalanine tracers. Values are means ± SE and are statistically not significantly different (P = 0.69).

Table 1. Leucine and phenylalanine enrichments during basal, postabsorptive conditions and feeding

<table>
<thead>
<tr>
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<th>Muscle Tissue Fluid</th>
<th>Muscle Proteins</th>
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reported in the literature, particularly those in a very similar study population (37). We found that the basal rate of mixed muscle protein FSR was 0.051 ± 0.004%/h with the phenylalanine tracer, and Volpi et al. (37) reported a value of 0.058 ± 0.005%/h for healthy elderly men and women. Nonetheless, there is a wide range of values for the basal rate of mixed muscle protein FSR in the human quadriceps in the literature; the source of this variability cannot readily be explained. Our data suggest that some of this variability may be due to the choice of tracer; however, this can only account for a small portion of the wide range in values reported. We determined that the difference in FSR that stems from the choice of tracer amino acid is ~20%, whereas literature values for mixed muscle protein FSR (obtained by using stable isotope-labeled leucine or phenylalanine tracers) vary twofold (from ~0.04 to 0.08%/h) even when restricted to reports that studied healthy young individuals during basal, postabsorptive conditions (11–13, 16, 23, 24, 36, 37, 41). The reliance of different precursor pools for the calculation of FSR may further add to the variability in results from different studies but is not expected to account for more than an additional 20–40% (2, 7, 8, 13, 38). Thus factors that are unrelated to the tracer and analyses employed must be responsible for the inconsistency in values found in the literature.

Feeding, as expected, stimulated muscle protein synthesis in our study, and the response (relative increase in FSR) was independent of the tracer used to measure the FSR. However, the feeding-induced increase in FSR in our study was small because of the age of our subjects and the small amount of food, particularly protein, provided. Amino acids stimulate muscle protein synthesis in a dose-dependent manner (5), and muscle of older adults is resistant to the anabolic effects of amino acids (10, 12, 35). Thus, although feeding or hyperaminoacidemia has often been found to cause a doubling or even greater increase in muscle protein FSR (5, 22, 35), we only found a ~20% increase in muscle protein FSR when providing ~10 g of protein; this compares well with a doubling of muscle protein FSR in older adults in response to oral administration of a mixed amino acid solution, containing a total of 40 g of amino acids given in small aliquots during 3 h (36).

The reasons for the discrepancy in the measured muscle protein synthesis rate with leucine and phenylalanine tracers in our study are not clear. Since the difference in FSR obtained with the leucine and phenylalanine tracer was independent of the phenylalanine isotope used, and we have ruled out differences due to potential errors in the preparation of enrichment standards and speculate that the discrepancy is due to the amino acid itself. In fact, Iresjö et al. (15) recently described a phenomenon that would fit this notion in cell culture, wherein phenylalanine stimulated the incorporation of a phenylalanine but not a tyrosine tracer into protein and vice versa. This was observed when large amounts of these amino acids (405 μM) were in the medium and could therefore be related to amino acid competition for transporters. In our study, we can rule out this mechanism because plasma amino acid availability is far below the concentrations that cause saturation of transmembrane leucine and phenylalanine transport (K_m ~ 20 mM) (14); furthermore, the phenylalanine and leucine transmembrane enrichment gradients in muscle were very similar, which would not be expected if there were selective inhibition of leucine or phenylalanine uptake into muscle. It is possible that the enrichments of phenylalanine and leucine in muscle tissue fluid, which we used as the precursor pool for calculating the FSR, do not provide equally good estimates of the respective aminoacyl-tRNA enrichment. It has been suggested that leucine exhibits functional compartmentalization within the muscle, with a pool of leucine directed toward oxidation and a separate pool directed toward protein synthesis (30). In contrast, phenylalanine is not oxidized within skeletal muscle (39); thus the phenylalanine enrichment in muscle tissue fluid probably reflects its tRNA charged pool better than the leucine enrichment in muscle tissue fluid, which represents a mixture of its different functional compartments. We did not directly measure the aminoacyl-tRNA enrichment, because the amino acid enrichment in muscle fluid has been shown to be a suitable surrogate (2, 18, 38). Furthermore, it requires a large amount of tissue (~1 g) to analyze the aminoacyl-tRNA (38), and it is technically very challenging to obtain accurate and robust results (38, 39). In fact, differences between amino acid labeling in muscle tissue fluid and aminoacyl-tRNA that could account for the differences in FSR obtained with leucine and phenylalanine tracers in our study are probably too small to be measured accurately. Most important of all, it is (for those reasons) usually not done. Furthermore, the conclusions from our study are the same even if we assume, as suggested by Lobley et al. (19), that the amino acid precursor enrichment in the extracellular space (blood) provides a closer approximation of the true precursor enrichment for muscle protein synthesis than an intracellular source. When we calculated the FSR with plasma leucine and phenylalanine enrichments as the precursors, FSR_{Phe} was also ~20% less than FSR_{Leu} (during fasting conditions, FSR_{Leu} was 0.044 ± 0.004%/h and FSR_{Phe} was 0.035 ± 0.003%/h, P < 0.01; during feeding, FSR_{Leu} was 0.059 ± 0.005%/h and FSR_{Phe} was 0.049 ± 0.004%/h, P < 0.02; means ± SE).

Interestingly, differences very similar to the ones in our study have been found by comparing the basal rate of muscle protein synthesis with the flooding dose technique (21) and when the basal rate of muscle protein synthesis was calculated from nonoxidative leucine and phenylalanine uptake with the classic two-pool arteriovenous balance model (3). McNurlan et al. (21) found that when using the flooding dose technique, the FSR determined with a leucine tracer is ~15% greater than the FSR determined with a phenylalanine tracer; the difference failed to reach statistical significance in some of the comparisons, most likely due to lack of statistical power. The arteriovenous balance approach used by Bennet et al. (3) is based on entirely different assumptions and is independent of the precursor pool issue discussed, which suggests that other factors also contribute to the amino acid tracer-dependent rate of muscle protein synthesis. However, unlike our study, the relative increase in muscle protein synthesis in response to hyperaminoacidemia was also more or less marked depending on the tracer used for evaluation of muscle protein metabolism with leucine and phenylalanine tracers in a limb arteriovenous balance model (i.e., greater with phenylalanine than leucine in the study by Bennet et al. (3) and greater with leucine than phenylalanine in the study by Tessari et al. (33)). This, however, could largely be due to errors in measuring limb leucine oxidation.

Preferential incorporation of leucine into fast-turning over muscle proteins (e.g., sarcoplasmic and mitochondrial proteins...
(1, 22) or, vice versa, preferential incorporation of phenylalanine into slower turning over proteins [e.g., myofibrillar proteins (1, 22)] could cause the apparent discrepancy in FSR. However, the relative contents of leucine and phenylalanine in the major muscle protein fractions (myofibrillar, sarcoplasmic and insoluble proteins) are very similar (9). It is therefore unlikely that such a phenomenon contributed significantly to the differences in FSR obtained by using a leucine versus a phenylalanine tracer. Thus, although our study leaves some questions concerning the potential source of discrepancy unanswered, it provides meaningful and important novel information concerning the use of leucine and phenylalanine tracers for the investigation of muscle protein metabolism.

In summary, we have demonstrated that there is a considerable and statistically highly significant difference in the rate of muscle protein synthesis obtained via constant infusion of leucine and phenylalanine tracers. However, the anabolic response to feeding is independent of the tracer used. Therefore, different precursor amino acid tracers cannot be used interchangeably for the evaluation of muscle protein synthesis. Furthermore, data from studies that applied different amino acid tracers to measure muscle protein FSR can only be compared quantitatively with respect to the relative changes in FSR due to anabolic stimuli such as hyperaminoacidemia or feeding, whereas comparison of absolute rates of protein synthesis is not valid.

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