Timing of the initial muscle biopsy does not affect the measured muscle protein fractional synthesis rate during basal, postabsorptive conditions

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Timing of the initial muscle biopsy does not affect the measured muscle protein fractional synthesis rate during basal, postabsorptive conditions

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Smith GI, Villareal DT, Lambert CP, Reeds DN, Mohammed BS, Mittendorfer B. Timing of the initial muscle biopsy does not affect the measured muscle protein fractional synthesis rate during basal, postabsorptive conditions. J Appl Physiol 108: 363–368, 2010. First published November 25, 2009; doi:10.1152/japplphysiol.00957.2009.—The muscle protein fractional synthesis rate (FSR) is determined by monitoring the incorporation of an amino acid tracer into muscle protein during a constant-rate intravenous tracer infusion. Commonly two sequential muscle biopsies are obtained some time after starting the tracer infusion. However, other protocols, including those with an initial biopsy before starting the tracer infusion to measure the background enrichment and those with only a single biopsy after several hours of tracer infusion have been used. To assess the validity of these approaches, we compared the muscle protein FSR obtained by calculating the difference in [ring-2H5]phenylalanine and [5,5,5-2H3]leucine incorporation into muscle protein at ~3.5 h after starting the tracer infusion and 1) at 60 min; 2) before starting the tracer infusion (background enrichment); 3) a population average muscle protein background enrichment; and 4) by measuring the tracer incorporation into muscle protein at ~3.5 h assuming essentially no background enrichment. Irrespective of the tracer used, the muscle protein FSR calculated from the difference in the muscle protein labeling several hours after starting the tracer infusion and either the labeling at 60 min or the background enrichment were not different (e.g., 0.049 ± 0.007%/h vs. 0.049 ± 0.007%/h, respectively, with [2H5]phenylalanine; P = 0.99). However, omitting the initial biopsy and assuming no background enrichment yielded average FSR values that were ~15% (with [2H5]phenylalanine) to 80% (with [2H3]leucine) greater (P ≲ 0.059); using a population average background enrichment reduced the difference to ~3% (P = 0.76) and 22% (P = 0.52) with [2H5]phenylalanine and [2H3]leucine, respectively. We conclude that during basal, postabsorptive conditions, valid muscle protein FSR values can be obtained irrespective of the timing of the initial biopsy so long as the protein labeling in two sequential biopsies is measured whereas the single biopsy approach should be avoided.

amino acid; muscle protein turnover

PROTEIN SYNTHESIS RATES in human muscles are commonly determined by measuring the difference in incorporation of a stable isotope-labeled amino acid tracer (usually phenylalanine or leucine) into muscle protein between two sequential biopsies during a primed, continuous intravenous infusion of the tracer (2, 13, 15, 25, 27, 35, 37, 44, 45, 48). The muscle protein fractional synthesis rate (FSR) is then calculated by dividing the increment in label incorporation over time by the labeling of the precursor for protein synthesis [ideally the labeling of the aminoaeryl-tRNA specific for the tracer amino acid infused, but a number of surrogates have been proposed and used (23, 46, 47)]. For this approach to be valid, it is necessary that label incorporation into proteins proceeds in a linear manner, which is the case if the precursor pool is steady during that time. Thus it has been recommended to administer the primed constant tracer infusion for at least 1 h before the first biopsy (47). Indeed many protocols have been designed with an initial muscle biopsy between 60 and 120 min after the start of the amino acid tracer infusion and a second one some 3 to 12 h later (7, 16, 19, 20, 26, 29, 30, 34, 41, 49). Recently, Volpi et al. (42) demonstrated that the protein labeling is linear between 120 and 360 min after starting a primed, constant phenylalanine tracer infusion, validating this approach. However, no data were provided for earlier or later time points, and the validity of studies in which the first biopsy was obtained as soon as 30 min after (2, 3) or immediately before starting the tracer infusion (6, 25, 27, 28, 32, 40) remains unclear. Theoretically these approaches might only introduce minimal error because studies in animals and human subjects have demonstrated rapid entry (within minutes) of intravenously administered labeled amino acids into the intracellular space and muscle proteins (1, 9, 38), which should ensure linearity of tracer incorporation almost instantly. However, this has never been carefully evaluated.

Moreover, several investigators have taken only a single biopsy several hours after starting the tracer infusion, apparently assuming no or only negligible background muscle protein enrichment in their calculation of the FSR (4, 14, 15, 17), or alternatively used published population average values for the background enrichment of muscle protein as the initial value in the FSR calculation (11, 12), as suggested by Rennie et al. (36). If valid FSRs can be obtained by using a single biopsy this would be advantageous to both investigators and study participant and useful in circumstances in which there might be reason to limit the number of biopsies on ethical grounds (e.g., certain patient groups) or when opportunities for an initial biopsy are limited (e.g., studies in patients intraoperatively).

The purpose of the present study was to 1) monitor during the early stages of a primed constant infusion of labeled leucine the plasma α-ketoisocaproate (α-KIC) labeling and the KIC-to-leucine enrichment ratio as an index of the equilibration of plasma and intracellular amino acid labeling, and 2) to evaluate how the timing, or omission, of the initial muscle biopsy affects the calculated FSR. Specifically, we measured in healthy men during basal, postabsorptive conditions 1) plasma leucine and α-KIC labeling before and at frequent time points immediately after the start of a primed, continuous [5,5,5-2H3]leucine infusion, and 2) the muscle protein FSR during primed, continuous intravenous infusions of [ring-2H5]phenylalanine or [5,5,5-2H3]leucine with muscle biopsies obtained either immediately before and several hours after starting the tracer infusion or 60 min and several hours after starting the tracer infusion.
Determine the leucine and phenylalanine labeling of muscle protein in lipoprotein metabolism studies that included a primed constant 
\[\text{sec} \times \text{H}11006\] and at 5, 15, 30, 60, 90, 120, 150, and 180 min after the start of the tracer infusion study. Blood samples (4 ml each) were obtained before and stored at 80°C until final analysis. Following anesthesia with 2% lidocaine solution, a cannula was inserted into an antecubital vein at 0600. At 0700, a primed constant infusion of \([5,5,5-\text{H}_2]\text{leucine (priming dose 7.2 }\mu\text{mol/kg body wt, infusion rate 0.12} \mu\text{mol/kg body wt, } \text{sec} \times \text{H}006\text{)}\) was started and maintained until completion of the study. Blood samples (4 ml each) were obtained before and at 5, 15, 30, 60, 90, 120, 150, and 180 min after the start of the tracer infusion.

In the 22 men studied to determine the effect of biopsy timing on the measured muscle protein FSR, a cannula was inserted into an antecubital vein at ~0600 for infusion of the stable isotope labeled amino acid precursors. Another one was inserted into a vein of the contralateral forearm for blood sampling. At ~0700, a primed constant infusion of \([5,5,5-\text{H}_2]\text{leucine (priming dose 2.03 ± 0.03} \mu\text{mol/kg body wt, infusion rate 0.055 ± 0.002} \mu\text{mol/kg body wt, } \text{sec} \times \text{H}005\text{)}\) was started and maintained until completion of the study. Blood samples (4 ml each) were obtained before and at 5, 15, 30, 60, 90, 120, 150, and 180 min after the start of the tracer infusion.

In the 5 men who were studied to determine the relationship between plasma leucine and α-KIC labeling early after the start of a leucine tracer infusion, a cannula was inserted into an antecubital vein at ~0600 for infusion of the stable isotope labeled amino acid precursors. Another one was inserted into a vein of the contralateral forearm for blood sampling. At ~0700, a primed constant infusion of \([5,5,5-\text{H}_2]\text{leucine (priming dose 0.03} \mu\text{mol/kg body wt, infusion rate 0.012} \mu\text{mol/kg body wt, } \text{sec} \times \text{H}012\text{)}\) was started and maintained until completion of the study. Blood samples (4 ml each) were obtained before and at 5, 15, 30, 60, 90, 120, 150, and 180 min after the start of the tracer infusion.

Experimental protocol. Subjects were instructed to adhere to their regular diet and refrain from vigorous exercise for 3 days before they were admitted to the clinical research unit on the evening before the metabolic study. At 2000 they consumed a standard meal providing 12 kcal/kg body wt; 55% of the total meal energy was provided as carbohydrates, 30% as fat, and 15% as protein. Subjects then rested in bed and fasted (except for water) until completion of the tracer infusion study the next day.

In the five men who were studied to determine the relationship between plasma leucine and α-KIC labeling early after the start of a leucine tracer infusion, a cannula was inserted into an antecubital vein at ~0600 for infusion of the stable isotope labeled amino acid precursors. Another one was inserted into a vein of the contralateral forearm for blood sampling. At ~0700, a primed constant infusion of \([5,5,5-\text{H}_2]\text{leucine (priming dose 7.2 }\mu\text{mol/kg body wt, infusion rate 0.12} \mu\text{mol/kg body wt, } \text{sec} \times \text{H}006\text{)}\) was started and maintained until completion of the study. Blood samples (4 ml each) were obtained before and at 5, 15, 30, 60, 90, 120, 150, and 180 min after the start of the tracer infusion.
average TTR of protein-bound phenylalanine over time (Fig. 2),
the lines connecting the TTR between 0 min and 210 min and 
60 min and 240 min, respectively, were superimposable; the 
intercept (0.000022 ± 0.000012) was significantly different 
from zero (P = 0.05) but similar to the average background 
TTR (0.000018) from a previously studied cohort (P = 0.75).
The muscle protein FSRs obtained by using the 
\(^{13} \text{Hs}\)phenylalanine tracer are presented in Fig. 3. The muscle 
protein FSR calculated from the labeled phenylalanine incorpo-
ration into protein between 0 min (measured TTR) and 210 min 
was not different (P = 0.99) from the FSR calculated from 
the label incorporation into protein between 60 min and 240 min.
Power analysis revealed that the sample size required to detect a 
difference of the observed magnitude with sufficient power 
(\(\geq 0.80\)) to reject the null hypothesis and a sufficiently small 
probability of Type-I error (\(\alpha < 0.05\)) is \(1\) million. Omitting the 
initial biopsy and assuming no phenylalanine labeling in muscle 
protein before the start of the tracer infusion gave FSR values that 
were on average \(\sim 15\% \ (0.009 \pm 0.005\%/h)\) greater (P = 0.059) 
than the FSR values obtained by measuring the labeling of muscle 
protein in two sequential biopsies whereas using the average 
background TTR from a previously studied cohort resulted in 
FSR values that were on average not different (\(\sim 3\%\) difference, 
P = 0.76) from those obtained from measuring the muscle protein 
labeling in both an initial and final biopsy. The coefficients of 
variation for each of these methods were similar: 33% (biopsies 
immediately before and at 210 min after the start of the tracer 
infusion), 34% (biopsies at 60 min and 240 min after the start of 
the tracer infusion), 27% (omitting the first biopsy and assuming 
zero background labeling), and 32% (omitting the first biopsy and 
using a population average background enrichment). The average 
values of the variables used to calculate the FSR (i.e., the muscle 
free phenylalanine labeling in the final biopsy, the change in 
muscle protein bound phenylalanine labeling over time, and the 
duration between two biopsies or the time lapse from beginning 
the tracer infusion and the final biopsy for the single biopsy 
approach) are presented in Table 1.

With the leucine tracer, the results were essentially the same, 
except that the results were more variable with the single-
biopsy approaches, most likely because of the greater natural
Table 1. Muscle free phenylalanine/leucine labeling in the final biopsy, the change in muscle protein bound phenylalanine/leucine labeling over time, and the duration between two biopsies or the time lapse from beginning the tracer infusion and the final biopsy for the single-biopsy approach

<table>
<thead>
<tr>
<th></th>
<th>Two Biopsies</th>
<th>Single Biopsy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 min and 210 min</td>
<td>60 min and 240 min</td>
</tr>
<tr>
<td>[ring-2H3]phenylalanine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Muscle free TTR</td>
<td>0.0639 ± 0.0037</td>
<td>0.0642 ± 0.0078</td>
</tr>
<tr>
<td>Time for FSR calculation, min</td>
<td>224 ± 4</td>
<td>171 ± 3</td>
</tr>
<tr>
<td>ΔMuscle protein TTR</td>
<td>0.000119 ± 0.000022</td>
<td>0.000094 ± 0.000222</td>
</tr>
<tr>
<td>[5,5,5-2H3]leucine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Muscle free TTR</td>
<td>0.0327 ± 0.0020</td>
<td>0.0530 ± 0.0078</td>
</tr>
<tr>
<td>Time for FSR calculation, min</td>
<td>218 ± 3</td>
<td>177 ± 4</td>
</tr>
<tr>
<td>ΔMuscle protein TTR</td>
<td>0.000046 ± 0.000005</td>
<td>0.000056 ± 0.000008</td>
</tr>
</tbody>
</table>

Values are means ± SE. TTR, tracer-to-tracee ratio; FSR, fractional synthesis rate.

abundance of [2H3]leucine compared with [2H5]phenylalanine in muscle proteins. The muscle free leucine labeling was not significantly different at 60 min and 240 min (TTR 0.0417 ± 0.0042 and 0.0530 ± 0.0078, respectively; P = 0.057). The muscle protein FSRs obtained by using the [2H3]leucine tracer are presented in Fig. 4. The FSR values obtained with two biopsies taken either before and 210 min after the start of the tracer infusion or at 60 and 240 min after the start of the tracer infusion were not different (P = 0.66). Power analysis revealed that the sample size required to detect a difference of the observed magnitude with sufficient power (≥0.80) to reject the null hypothesis and a sufficiently small probability of Type-I error (α < 0.05) is ~160 (assuming the smaller SD) and ~269 (assuming the larger SD). Omitting the initial biopsy and assuming no background leucine enrichment in muscle protein before the start of the tracer infusion gave FSR values that were on average ~80% (0.0031 ± 0.0012%/h, P = 0.03) greater than the FSR values obtained by measuring the labeling of muscle protein in two sequential biopsies. This difference was significantly greater than the corresponding value obtained when using the phenylalanine tracer (P = 0.05). The FSR values obtained by using the average background [2H3]leucine enrichment in muscle protein from a previously studied cohort (TTR = 0.00056) were on average ~22% different (P = 0.52) from those obtained by measuring the muscle protein labeling in two sequential biopsies. The coefficients of variations for each of these methods, in ascending order, were 16% with biopsies at 60 min and 240 min after the start of the tracer infusion, 25% with biopsies immediately before and at 210 min after the start of the tracer infusion, 59% when omitting the first biopsy and assuming zero background labeling, and 145% when omitting the first biopsy and using a population average background enrichment. The average values of the variables used to calculate the FSR (i.e., the muscle free leucine labeling in the final biopsy, the change in muscle protein bound leucine labeling over time, and the duration between two biopsies or the time lapse from beginning the tracer infusion and the final biopsy for the single-biopsy approach) are presented in Table 1.

DISCUSSION

In the present study we compared the calculated basal skeletal muscle protein FSR obtained by using different experimental protocols for measuring the rate of label incorporation into muscle protein during a primed constant intravenous infusion of a tracer amino acid. Specifically we examined whether an initial muscle biopsy is necessary and whether the timing of the initial biopsy affects the calculated muscle protein FSR. We found that similar muscle protein synthesis rates are obtained irrespective of whether the initial muscle biopsy is taken immediately before or 60 min after starting a primed, constant amino acid tracer infusion, suggesting that the timing of the initial biopsy does not affect the final FSR value. However, omitting the initial biopsy and either assuming no background enrichment or using a population average background enrichment yields average FSR values that are greater than those based on two biopsies, with the magnitude of error dependent on the amino acid tracer used.

The fact that independent of the tracer used the FSR measured between 0 and 210 min after starting the tracer infusion was not different from the FSR measured between 60 and 240 min after starting the tracer infusion indicates that either the major underlying assumption for the FSR approach (i.e., linear tracer incorporation) was met or it was violated only to a minimal and undetectable extent, which did not affect the
results. This implies that the tracer labeled the aminoacyl tRNA pool very rapidly and was incorporated into muscle protein almost instantaneously after starting the infusion. We indeed observed rapid equilibration between the plasma leucine and α-KIC labeling and near steady-state labeling of the KIC pool within 15–30 min after the leucine tracer administration in the present study. This is consistent with earlier work that demonstrated that 70% of a phenylalanine tracer given intravenously in rats appeared in muscle within 3 min and tracer incorporation into muscle protein occurred as soon as 2 min after administration (1, 38) and that the half-life of methionine (which is similar in its metabolism and transport to the branched-chain amino acids) uptake in human muscle is 6 min (9). The FSR values obtained by using two biopsies are in good agreement with those previously reported by us and other investigators. For example, we have previously reported that in young and middle-aged healthy adults in the postabsorptive state at rest the mixed muscle protein FSR measured by using a [2H3]phenylalanine tracer is ~0.045%/h (39); most others who use a phenylalanine tracer and two biopsies report average values in the range of 0.04%/h to 0.06%/h (e.g., 2, 8, 21, 24, 33, 45) although some reports include somewhat smaller or greater values (e.g., 2, 5, 10, 18). This strengthens our confidence that the data obtained are robust and that it is possible to accurately measure the muscle protein FSR within a few hours of the start of the tracer infusion without the need for an “equilibration period” before the initial biopsy.

The degree to which the average FSR deviates from the “true” FSR when relying on a single biopsy measurement depends on the choice of tracer, the derivative for MS analysis, and the labeling value used as a surrogate for the background enrichment of muscle protein. Relatively small errors result when using a tracer and derivative pair with a low natural abundance of the analyzed isotope and assuming essentially no background enrichment (i.e., when using a [2H3]phenylalanine tracer the average FSR values fell within ~15% of the actual FSR) whereas the average error can be in excess of 50% when using a tracer and derivative with a high natural abundance (i.e., when using a [2H3]leucine tracer the average FSR values were ~80% different from the actual FSR). In contrast, the use of a population average background enrichment when omitting the initial biopsy reduces, but does not entirely abolish, this error.

These findings have important implications not only for the design of studies in which the basal muscle protein FSR at rest is to be determined but also studies in which the muscle protein FSR will be determined under conditions other than fasting at rest (e.g., postexercise or during feeding) or studies with a sequential study design (e.g., a basal period followed by feeding, etc.). First, it is unlikely that the conclusions from our study would have been different had we made our measurements during conditions other than the postabsorptive state, at rest (e.g., after exercise or during intravenous nutrient infusion) unless there were reason to believe that the time it takes to reach isotopic equilibrium between the plasma and intracellular pool were greatly extended (which could adversely affect the result) by these circumstances. Second, our findings suggest (although we do not have direct proof for this) that sequential study designs, particularly those in which care is taken to minimize changes in the precursor pool enrichment (e.g., 3, 39, 43), are most likely valid because we have demonstrated that small initial temporary imbalances in the precursor pool enrichment do not affect the measured muscle protein FSR.

In summary, the plasma and intracellular amino acid pools equilibrate quickly during a primed, continuous intravenous amino acid tracer infusion, and valid basal muscle protein FSR values can be obtained irrespective of the timing of the initial biopsy when the protein labeling in two sequential biopsies is measured. Under some circumstances valid average muscle protein FSR values can be obtained by measuring the labeling of protein in a single biopsy after some time of continuous tracer infusion, although this approach could potentially result in highly erroneous results and should therefore be avoided.

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DISCLOSURES

None of the authors had conflicts of interest.

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