Aminoacyl-tRNA enrichment after a flood of labeled phenylalanine: insulin effect on muscle protein synthesis

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Muscle protein synthesis in dogs measured by flooding with L-[2H5]phenylalanine (70 mg/kg) was significantly stimulated by infusion of insulin with amino acids. The stimulation of muscle protein synthesis was similar when calculated from enrichment of L-[2H5]phenylalanine (54±10%, P<0.001), plasma phenylalanine (61±10%, P<0.001), or tissue fluid phenylalanine (54±10%, P<0.001). The time course for changes in enrichment of L-[2H5]phenylalanine throughout the flooding period was determined for plasma, tissue fluid, and phenylalanyl-tRNA in the basal state and during the infusion of insulin with amino acids. Enrichments of plasma free phenylalanine and phenylalanyl-tRNA were equalized between 20 and 45 min, although the enrichment of phenylalanyl-tRNA was lower at early time points. Rates of muscle protein synthesis obtained with the flooding method and calculated from plasma phenylalanine enrichment were comparable to those calculated from phenylalanyl-tRNA and also to those obtained previously with a continuous infusion of phenylalanine with phenylalanyl-tRNA as precursor. This study confirms that, with a bolus injection of labeled phenylalanine, the enrichment of aminoacyl-tRNA, the true precursor pool for protein synthesis, can be assessed from more readily sampled plasma phenylalanine.

amino acids; L-[2H5]phenylalanine; dog; flooding technique; ketoisocaproic acid; stable isotopes

INSULIN IS GENERALLY CONSIDERED an anabolic hormone, with reported stimulation of protein synthesis in vitro and in vivo (18, 32, 34). However, its action on human muscle is not completely clear. Several studies in human subjects have demonstrated that the main effect of insulin is to inhibit protein degradation (16, 22, 26, 36, 38), but whether this is accompanied by a stimulation of protein synthesis is still controversial. Many studies have failed to show any stimulation of muscle protein synthesis by insulin (16, 22, 36, 38). However, some studies indicate that insulin does stimulate muscle protein synthesis in humans (3, 4, 42).

In most of the studies in humans, muscle protein synthesis was measured with a constant infusion of a labeled amino acid. These measurements rely on an accurate estimate of the enrichment of the immediate precursor for protein synthesis. However, the true precursor, aminoacyl-tRNA, is difficult to determine routinely, and consequently it is often estimated from the enrichment of more readily accessible amino acid pools, such as the free amino acid in plasma or within the tissue (tissue fluid). The use of surrogate pools to estimate precursor labeling is based on the assumption that the experimental treatment does not alter the relationship between the labeling of the sampled pool and that of aminoacyl-tRNA. Because insulin affects amino acid transport in muscle (23) and can inhibit muscle protein degradation (16, 22, 26, 36), studies with insulin have the potential to alter precursor enrichment either by affecting the amount of labeled amino acid entering the cell or by decreasing the contribution of unlabeled amino acids derived from protein degradation to the charging of aminoacyl-tRNA. Providing exogenous amino acids can also alter the relationship of enrichment within surrogate amino acid pools to the true precursor. For example, increasing the plasma concentration of the amino acid used to trace protein synthesis (i.e., the tracer) twofold during the infusion of insulin and amino acids in dogs altered the relationship of the tracer enrichment in the plasma to the enrichment in aminoacyl-tRNA (9). This alteration resulted in an overestimate of the effect of insulin on muscle protein synthesis when the plasma enrichment of tracer was used to estimate muscle protein synthesis (9).

However, the problem of accurately determining precursor enrichment may be minimized by the flooding technique. With this method, a labeled amino acid is injected not in tracer amounts, but as a large bolus, with the aim of flooding all amino acid pools to a similar enrichment (e.g., Refs. 11, 13, and 25). With

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this technique, the labeling of aminoacyl-tRNA is less likely to be affected by experimental manipulations, so that assessment of enrichment from either the plasma or the free amino acid within the tissue provides an accurate estimate of the enrichment of the precursor for protein synthesis (see review in Ref. 19).

The aim of the present study was to investigate the effect of insulin on muscle protein synthesis with the flooding technique in a dog model that allowed comparison of the direct measurement of the enrichment of aminoacyl-tRNA and the enrichment of tracer in potential surrogate pools (free amino acid in plasma and tissue fluid) under conditions of altered protein degradation and amino acid uptake. Muscle protein synthesis was measured with L-[2H5]phenylalanine (at 70 mg/kg) in a basal (postabsorptive) state and during the infusion of sufficient insulin to increase plasma insulin concentrations to levels comparable to those during feeding. To compensate for the insulin-induced decline in plasma glucose and amino acids (17), an amino acid mixture and glucose were also infused. The time course of labeling of phenylalanyl-tRNA after a flooding amount of L-[2H5]phenylalanine and the effect of a flood of phenylalanine on the labeling of other simultaneously infused amino acids were also investigated.

METHODS

Two groups of eight conditioned adult male dogs of mixed breed (wt 20 ± 2 kg) were used in the experiments. Animals were singly housed and fed a standard diet (Purina Lab Canine Chow, Purina Mills, Richmond, IN) with free access to food from 0830 to 1300 and to water throughout the day. Each dog adapted to the laboratory environment for ≥7 days before the experiments were begun.

Experimental Protocol

The synthesis of muscle protein was measured during a 3-h basal period and again during infusion of both insulin and an amino acid mixture to maintain euaminoacidemia (Figs. 1 and 2).

At 7:30 AM, acepromazine (0.15 mg/kg) was given, and cannulas were inserted in two contralateral leg veins. Dogs were then anesthetized with pentobarbital and intubated. Anesthesia was maintained throughout the experiment by mechanical ventilation with O2 containing 1.5–2% isoflurane. A cannula was inserted in the carotid artery, and samples were taken at 30-min intervals for the measurement of plasma free amino acid levels, insulin concentrations, and blood gases. The ventilation rate was continually adjusted to maintain blood pH at initial values, because muscle protein synthesis is sensitive to pH (10). Starting at 3 h, a euglycemic hyperinsulinemic clamp (12) was performed for a further 3 h (Figs. 1 and 2). Insulin (Eli Lilly, Indianapolis, IN) was infused at a constant rate of 1 mU·kg⁻¹·h⁻¹. Plasma glucose concentration was closely monitored, and glucose was infused at a variable rate to maintain plasma glucose concentration at basal levels. A commercially available solution containing 10% amino acids (TrophAmine, Braun Medical, Irvine, CA) was also infused at 0.42 ml·kg⁻¹·h⁻¹ (6.72 mg N·kg⁻¹·h⁻¹) for 3–6 h (Figs. 1 and 2). All protocols were approved by the Institutional Animal Care and Use Committee of the State University of New York at Stony Brook.

**Experiment 1. Effect of insulin on the rate of muscle protein synthesis.** In this experiment, muscle protein synthesis was measured with the flooding technique under basal conditions (2.25–3 h) and during the infusion of insulin with glucose and amino acids to prevent insulin-induced reductions in plasma insulin levels (5.25–6 h) (Fig. 1). L-[2H5]phenylalanine (MassTrace, Woburn, MA) and unlabeled L-phenylalanine (Ajinomoto, Tokyo, Japan) were injected over a 10-min period at 70 mg/kg and 10 mole percent excess (MPE) for the first injection and 20 MPE for the second. Blood samples were taken at intervals to measure changes in the enrichment of plasma phenylalanine. Forty-five minutes after the injection of the labeled phenylalanine, a muscle biopsy was taken from the biceps femoris muscle for the measurement of isotope incorporation into muscle protein and for the determination of phenylalanine enrichment within the tissue. For the second measurement of muscle protein synthesis, a biopsy was taken before the injection of L-[2H5]phenylalanine and at the end of the 45-min incorporation period (Fig. 1). Muscle samples were snap-frozen in isopentane in a liquid nitrogen bath and stored at −70°C until analysis.

**Experiment 2. Effect of insulin and amino acid infusion on the time course of precursor and protein labeling with L-[2H5]phenylalanine flooding.** This experiment was designed to evaluate the labeling of intracellular precursor pools for protein synthesis after a bolus injection of L-[2H5]phenylalanine. Effects of the bolus injection of labeled phenylalanine on the labeling of phenylalanine precursor pools were investigated along with two other la-
beled amino acids, L-[1-13C]leucine and L-[2-15N]lysine (MassTrace), which were given as a priming amount of 18 μmol/kg and then infused at 24 μmol·kg⁻¹·h⁻¹ for the entire 6-h period (Fig. 2). As in experiment 1, L-[2H₅]phenylalanine was given at 2.25 (basal) and 5.25 h (during insulin and amino acid infusion). To ensure sufficient labeling of samples of muscle protein from early time points, the enrichment of the injection solution was increased to 20 MPE in the first and to 40 MPE in the second flood. Muscle biopsies of ~1 g were taken before and 5, 10, 20, and 45 min after the injection of L-[2H₅]phenylalanine (Fig. 2). Muscle biopsies were taken alternately from right and left hind legs, with care taken to avoid muscle fibers damaged from previous sampling. After the removal of each sample of muscle tissue, the leg was sutured and covered to preserve body temperature.

Analytical Methods

Enrichment of tissue protein. Tissue powdered in liquid N₂ was precipitated with cold perchloric acid (20 g/l) to separate free and protein-bound amino acids. Muscle protein was extensively washed with cold perchloric acid (20 g/l) and hydrolyzed with 6 M HCl (38). The enrichment of L-[2H₅]phenylalanine in protein was measured as previously described (39) with an MD800 gas chromatograph-mass spectrometer (GC-MS; Fisons Instruments, Beverly, MA), operated under electron impact conditions (38). Exchange of plasma samples and labeled standards between Stony Brook and Rochester ensured comparability of measurements.

Enrichment of aminoacyl-tRNA. Aminoacyl-tRNA was isolated as previously described (35). Briefly, aminoacyl-tRNA was extracted with phenol, precipitated with ethanol, deacylated by addition of 50 mM NaHCO₃, and incubated at 37°C for 2 h. After acidification, the amino acid solution was evaporated to dryness and resuspended in 0.01 M HCl. Amino acids were derivatized to their N-trifluoroacetyl iso-propyl esters, and the isotopic enrichments were determined by a GC-MS (Hewlett-Packard) operated under selected ion monitoring and positive ion chemical ionization conditions, as previously described (35). The ions at m/z 326 and 321 were monitored for phenylalanine.

Substrate and hormone analysis. Plasma glucose concentrations were determined with a Beckman Glucose Analyzer 2 (Beckman Instruments, Brea, CA). Plasma amino acid concentrations were measured by HPLC (Waters 2690, Milford, MA) after acetanilide precipitation of plasma protein with fluorescence detection of the o-phthaldehyde amino acid derivative. 6-Amino-n-caproic acid was used as an internal standard.

Plasma insulin was determined by radioimmunoassay (Diagnostic Products, Los Angeles, CA).

Calculations

The rates of muscle protein synthesis were calculated as fractional synthesis rates (FSR, i.e., the percentage of the muscle protein pool synthesized per day), as described by McNurlan et al. (40)

\[
FSR(\%/day) = \frac{E_{p1} - E_{p0}}{A \cdot t} \cdot 100
\]

where \(E_{p1} - E_{p0}\) represents the increase in enrichment of the protein-bound phenylalanine between the beginning and the end of the incorporation periods, \(A\) indicates the area under the curve of the precursor enrichment over the same period of time, and \(t\) is the time of incorporation expressed in days. The area \(A\) was calculated from the change in enrichment of plasma, tissue fluid, or aminoacyl-tRNA vs. time. The rates of
muscle protein synthesis under basal conditions were determined between 2.25 and 3 h. The rate of muscle protein synthesis during infusion of insulin and amino acids was determined between 5.25 and 6 h (Figs. 1 and 2).

Statistics
All of the results are expressed as means ± SE. Comparison of measurements in the basal state and with insulin and amino acid infusion were analyzed by a Student’s t-test for paired data. Comparison of three or more groups was made by an one-way analysis of variance (ANOVA). A P value <0.05 was considered statistically significant.

RESULTS

Plasma Insulin, Glucose, and Amino Acids

Both experiments 1 and 2 consisted of a basal period of 3 h followed by a 3-h period when insulin was infused with glucose and amino acids to ensure that concentrations did not fall below the basal values. Plasma insulin concentrations in the basal period were 31.1 ± 3.8 pmol/l in experiment 1 and 32.3 ± 5.6 pmol/l in experiment 2. They rose to 330 ± 30 and 316 ± 22 pmol/l, respectively, during the insulin infusion period (basal vs. insulin, P < 0.001), to levels corresponding to normal fed plasma concentrations (Fig. 3). Plasma glucose concentrations were maintained at basal levels during the infusion of insulin and amino acids (5.7 ± 0.2 vs. 5.4 ± 0.2 mmol/l in experiment 1 and 5.9 ± 0.2 vs. 5.6 ± 0.2 mmol/l in experiment 2).

The administration of the amino acid mixture (Troph-Amine) prevented the decline in plasma concentration of leucine (Fig. 3) and of many, but not all, amino acids during the infusion of insulin. In both experiments, plasma lysine, valine, arginine, threonine, and asparagine concentrations decreased during the infusion of insulin (P < 0.05) (Table 1). A decline in methionine and tryptophan plasma concentrations during the infusion of insulin was detected only in experiment 1 (P < 0.05; Table 1).

As expected, the levels of plasma phenylalanine were significantly elevated after injection of 70 mg/kg phenylalanine. Basal concentrations of phenylalanine in plasma were 43 ± 5 μmol/l (experiment 1) and 45 ± 2 μmol/l (experiment 2), rising to 165 ± 19 and 173 ± 6 μmol/l, respectively, 45 min after the injection of the flooding amount of phenylalanine. During the infusion of insulin and amino acids, the plasma phenylalanine concentration slowly declined, to reach a value of 67 ± 8 μmol/l in experiment 1 and 85 ± 4 μmol/l in experiment 2 at 5.25 h, just before the second bolus injection of phenylalanine. At 6 h, after the injection of the second flood of phenylalanine, plasma phenylalanine concentrations were 163 ± 19 (experiment 1) and 217 ± 14 μmol/l (experiment 2).

Effect of Insulin on Muscle Protein Synthesis

All but one dog in experiment 1 showed a stimulation in the rate of muscle protein synthesis with insulin and amino acid infusion, with an average increase of 52 ± 19% over the basal rates with plasma phenylalanine as the precursor (P = 0.0068) (Table 2, flooding method 1). Similarly, in experiment 2, the rate of muscle protein synthesis calculated from plasma phenylalanine enrichment was stimulated by 54 ± 10% by infusion of insulin and amino acids (P = 0.0004) (Table 2, flooding method 2).

In this study, all measurements of muscle protein synthesis were made on anesthetized dogs because of the need for large samples of muscle tissue throughout the experimental period. Although there is evidence that whole body protein synthesis is decreased in anesthetized dogs (29, 30), studies in rodents (27) and surgical patients (15) indicate that rates of muscle protein synthesis during anesthesia are comparable to...
measurements made during conscious conditions. More importantly, adequate ventilation is necessary to maintain blood pH during anesthesia (9, 10). The ability of anesthesia to alter the response of muscle protein synthesis to insulin was not specifically investigated in the present study.

**Effect of Insulin on Protein and Precursor Enrichments**

Muscle protein enrichment. Insulin did not alter the almost linear incorporation of L-[2H5]phenylalanine into muscle protein. Figure 4 shows that the increase of L-[2H5]phenylalanine enrichment within muscle protein was linear in both the basal period \((r^2 = 0.999)\) (Fig. 4A) and during the infusion of insulin \((r^2 = 0.997)\) (Fig. 4B). The linear incorporation in Fig. 4, A and B, also suggests that the rate of muscle protein synthesis was constant over both experimental periods despite the sequential removal of muscle tissue.

Precursor enrichment (experiment 1). The enrichment of L-[2H5]phenylalanine in plasma reached a peak value equivalent to 90% of the enrichment of the injected phenylalanine by 10 min after the injection, declining almost linearly thereafter (Fig. 5). At the end of the 45-min incorporation period, the average enrichment of free phenylalanine in the tissue fluid was similar to that of plasma after both the first bolus injection \((6.24 \pm 0.15 \text{ vs. } 6.17 \pm 0.07 \text{ MPE})\) and

![Graph showing time course of incorporation of L-[2H5]phenylalanine into muscle protein during the first (A) and second (B) bolus injection of L-[2H5]phenylalanine (70 mg/kg) in experiment 2. Enrichment of injected solution was 20 mole percent excess (MPE) in A and 40 MPE in B. Values are means ± SE; \(n = 8\).](image)

Table 2. Response of muscle protein synthesis to insulin plus amino acids

<table>
<thead>
<tr>
<th></th>
<th>Plasma</th>
<th>Tissue Fluid</th>
<th>tRNA</th>
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<tbody>
<tr>
<td><strong>Basal</strong></td>
<td>+ Insulin</td>
<td>+ Insulin</td>
<td>+ Insulin</td>
</tr>
<tr>
<td><strong>Flooding method 1</strong></td>
<td>2.05 ± 0.18</td>
<td>3.16 ± 0.43*</td>
<td>2.33 ± 0.49</td>
</tr>
<tr>
<td><strong>Flooding method 2</strong></td>
<td>2.34 ± 0.22</td>
<td>3.56 ± 0.34*</td>
<td>2.28 ± 0.20</td>
</tr>
<tr>
<td><strong>Constant infusion</strong></td>
<td>1.64 ± 0.19</td>
<td>2.39 ± 0.19*</td>
<td>2.73 ± 0.37</td>
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Values are means ± SE (\(n = 8\) for flooding methods 1 and 2, and \(n = 7\) for constant infusion). Rates of muscle protein synthesis (%/day) were determined from incorporation of L-[2H5]phenylalanine after a bolus injection of 70 mg/kg in experiment 1 (Flooding method 1) and experiment 2 (Flooding method 2) or a constant infusion of 12 µmol·kg⁻¹·h⁻¹ (constant infusion) (9) into biceps femoris muscle. Estimates of protein synthesis calculated from plasma Phe enrichment use the mean enrichment over the 45-min incorporation period. Estimates with tissue fluid and phenylalanyl-tRNA use the value obtained at end of incorporation (Flooding method 1) or the mean value for the entire time period (Flooding method 2). *Significantly different from corresponding basal values, \(P < 0.05\). Basal rates from both methods and all precursors, excluding those from constant infusion with plasma, are not significantly different (ANOVA). Rates with insulin and amino acids from both methods and all precursors, excluding those from constant infusion with plasma, are not significantly different (ANOVA).
the second bolus injection (11.80 ± 0.24 vs. 11.43 ± 0.26 MPE) (Fig. 5). The enrichment of the phenylallyl-tRNA was also similar to that of plasma 45 min after the first injection (ratio of Phe-tRNA to plasma enrichment, 0.91 ± 0.05) and at the end of the second injection (ratio of Phe-tRNA to plasma enrichment, 0.96 ± 0.08) (Fig. 5), indicating that the plasma enrichment of phenylalanine was a good approximation of the enrichment of phenylalanine at the site of protein synthesis. This similarity of enrichment is reflected in the similarity of the rate of protein synthesis calculated with phenylalanine in plasma, tissue fluid, or phenylallyl-tRNA (Table 2, Flooding method 1), with no significant difference among the estimates of muscle protein synthesis from the three pools in either the basal or insulin-stimulated rate (ANOVA).

**Precursor enrichment over time (experiment 2).** The relationship between enrichment of phenylalanine in plasma and aminoacyl-tRNA was investigated further in experiment 2, where biopsies were taken throughout the time course of the incorporation period. Figure 6 shows the enrichment of phenylalanine in phenylallyl-tRNA, plasma, and tissue fluid during the basal period and during the infusion of insulin with glucose and amino acids. As Fig. 6 demonstrates, phenylalanine enrichment within the tissue did not immediately reach the plasma value. At 10 min after the injection of L-[2H5]phenylalanine, the enrichment of phenylalanine within the tissue was 10% lower than that in plasma in the basal period and 15% lower than plasma enrichment during the infusion of insulin and amino acids (enrichment tissue fluid vs. plasma, \( P < 0.02 \)). However, the enrichment of tissue phenylalanine declined more slowly than that of plasma phenylalanine and was 14% higher than plasma at 45 min (enrichment tissue fluid vs. plasma, \( P < 0.02 \)) in the basal period and 8% higher at the end of the incorporation period during the infusion of insulin (enrichment tissue fluid vs. plasma, \( P < 0.02 \)). Consequently, the area under the enrichment vs. time curve, necessary for the calculation of the rate of muscle protein synthesis, was similar for tissue fluid and for plasma (665 ± 15 vs. 645 ± 12 in the first flood and 1,206 ± 31 vs. 1,242 ± 18 in the second flood, \( P = \text{not significant (NS)} \)), with the lower enrichment in the free tissue phenylalanine in the first part of the flood balanced out by the higher enrichment in the last 25 min.

The increase in enrichment of phenylallyl-tRNA was somewhat slower than that of the plasma and tissue fluid phenylalanine, reaching a maximal value between 10 and 20 min. The enrichment of phenylallyl-tRNA declined only slightly during the 45-min incorporation period (Fig. 6). As in experiment 1, at the end of the incorporation period the phenylallyl-tRNA enrichment was similar to that of plasma (11.88 ± 0.36 vs. 12.20 ± 0.35 during the first flood and 22.70 ± 0.72 vs. 24.39 ± 0.66 MPE in the second flood, \( P = \text{NS} \)). However, the area under the enrichment vs. time curve for phenylallyl-tRNA was 16 ± 3% lower than that for plasma in the first flood (543 ± 23 vs. 645 ± 12, \( P < 0.001 \)) and 19 ± 3% lower in the second flood (1,004 ± 43 vs. 1,242 ± 18, \( P < 0.001 \)).

The impact of the differences in labeling of amino acid pools used to represent the precursor for protein synthesis on the muscle protein synthesis rates is shown in Table 2. Rates of muscle protein synthesis calculated from the increase in phenylalanine enrichment in protein and the time course of enrichment of phenylalanine in plasma, tissue fluid, or phenylallyl-tRNA (Flooding method 2) are shown compared with the values from experiment 1 (Flooding method 1).
higher enrichment of phenylalanine in plasma and tissue means that the synthesis rates calculated from either plasma or tissue fluid were somewhat lower than those calculated with the true precursor, phenylalanyl-tRNA (Table 2). However, the degree of stimulation of protein synthesis observed with insulin and amino acids was similar when calculated from any of the phenylalanine pools. An increase of 54 ± 10% over basal levels was observed from plasma enrichment (basal vs. insulin, P = 0.0004). When tissue fluid was used to estimate the precursor enrichment, the stimulation of muscle protein synthesis rate was 61 ± 10% (basal vs. insulin, P = 0.0004); with phenylalanyl-tRNA, the stimulation was the same, 61 ± 10% (basal vs. insulin, P = 0.001) (Table 2, Flooding method 2; ANOVA for degree of stimulation, P = NS).

**Enrichment of simultaneously infused tracers.** In addition to assessing the time course of changes in enrichment of L-[2H5]phenylalanine during the flooding protocol, experiment 2 was also designed to ascertain whether flooding with phenylalanine altered the enrichment of two simultaneously infused labeled amino acids given in tracer amounts as a continuous infusion. Figure 7 shows the enrichment of L-[1-13C]leucine and L-[15N2]lysine given by continuous infusion during the bolus injection of phenylalanine.

Unlike phenylalanine, where the bolus injection facilitated the uptake of phenylalanine into the tissue, the enrichment of the other two tracers within the tissue remained much lower than the plasma enrichment. The average leucyl-tRNA enrichment was 59% of that of plasma free leucine in the basal state (7.93 ± 0.45 vs. 13.37 ± 0.68 MPE, P < 0.001) and 63% during the infusion of insulin and amino acids (8.67 ± 0.34 vs. 13.82 ± 0.78 MPE, P < 0.001) (Fig. 7A). For leucine, the enrichment of the deamination product KIC in plasma has also been used as a surrogate for the enrichment of leucine within the muscle tissue (37). The average enrichment of KIC in plasma was significantly higher than that of leucyl-tRNA in the basal state (9.97 ± 0.65 vs. 7.93 ± 0.45 MPE, P < 0.05), although the enrichment in the two pools tended to become closer during the infusion of insulin and amino acids (9.56 ± 0.49 vs. 8.67 ± 0.34 MPE, P < 0.05). The ratio between the enrichment of aminoacyl-tRNA and plasma was even lower for lysine. The average lysyl-tRNA enrichment was only 37% of that of plasma lysine during the basal period (5.77 ± 0.33 vs. 16.11 ± 0.97 MPE, P < 0.001) and 33% during insulin and amino acid infusion (6.87 ± 0.35 vs. 21.54 ± 1.10 MPE, P < 0.001).

Average leucyl-tRNA enrichment was similar to that of free leucine in the tissue fluid during the basal state (7.93 ± 0.45 vs. 8.39 ± 0.28 MPE) but tended to be lower during the treatment period (8.67 ± 0.34 vs. 9.20 ± 0.39 MPE, P = 0.067).

Unlike leucine, where the enrichment in tissue fluid was similar to that of leucyl-tRNA, the average lysine enrichment in the tissue fluid was significantly higher than that of lysyl-tRNA (basal: 5.77 ± 0.33 vs. 8.33 ± 0.44 MPE; insulin: 6.87 ± 0.35 vs. 10.84 ± 0.71 MPE; P < 0.001; Fig. 7B).

Interestingly, the injection of the large amount of phenylalanine resulted in a transient increase in both the leucyl- and lysyl-tRNA enrichment. During the first flood, leucyl-tRNA enrichment increased by ~12% at 5 min (P < 0.05) and lysyl-tRNA by ~14% (P = 0.1), returning to baseline levels at 20 min (Fig. 7). However, the rise in enrichment of intracellular pools was minimal during the second flood (Fig. 7).

**DISCUSSION**

This study was undertaken to determine the effect of insulin with provision of amino acids and glucose on the rate of muscle protein synthesis in postabsorptive, adult, nondiabetic dogs. The results show that the infusion of insulin with amino acids significantly stimulated the rate of muscle protein synthesis. Calculated in the usual way with plasma phenylalanine enrichment, the increase in muscle protein synthesis was
highly significant (53 ± 8% over the basal levels, experiments 1 and 2, n = 16, P < 0.001).

Although aminocyl-tRNA is the true precursor for protein synthesis, the low concentration of aminocyl-
tRNA within tissue makes the routine measurement of the enrichment of aminocyl-tRNA impractical because of the necessity for large (0.5–1 g) tissue samples. The flooding technique, with a bolus injection of a labeled amino acid, has been developed to flood all amino acid pools to similar enrichment, thereby minimizing the differences between the enrichment of the aminocyl-tRNA and the more easily accessible plasma amino acid pool. This study tested the assumption that the flooding method allows an accurate estimate of protein synthesis without the necessity for assessing the enrichment of aminocyl-tRNA.

To verify that the difference in enrichment between the true precursor for protein synthesis and the more readily sampled plasma pool was indeed minimized with the bolus injection of amino acid, an anesthetized dog model was employed so that large tissue samples could be taken for the isolation and direct measurement of enrichment in the aminocyl-tRNA pool. The results confirmed the equilibration between the enrichment of phenylalanine in plasma and phenylalanyl-tRNA at the end of the flooding period both in the basal state and during the infusion of insulin (Fig. 5), in agreement with the findings of Davis et al. (11) in neonatal pigs. However, Fig. 6 shows that there was equivalence of enrichment in plasma phenylalanine and phenylalanyl-tRNA for the interval 20–45 min after the injection of 70 mg/kg of l-[3H]phenylalanine, but before 20 min, the increase in the enrichment of phenylalanyl-tRNA was lower than that of plasma phenylalanine. However, because the plasma enrichment also declined faster than aminocyl-tRNA enrichment (Fig. 6), the area under the plasma curve became progressively closer to the area estimated from phenylalanyl-tRNA for longer periods of incorporation. Therefore, the estimate of protein synthesis rate from plasma phenylalanine enrichment was closer to the true value with increasing periods of incorporation. Moreover, had the incorporation period been prolonged to 90 min, as used in human studies, the difference between synthesis rates based on plasma or on aminocyl-tRNA would have been even less.

Although the amount of phenylalanine administered in dogs (70 mg/kg) was larger than that used in human volunteers (45 mg/kg), it is possible that more rapid equilibrium of plasma phenylalanine and phenylalanyl-tRNA might have been achieved if an even larger bolus injection of phenylalanine had been used. In human volunteers, increasing the amount of l-[3H]phenylalanine from 29 to 45 mg/kg improved the ratio between the enrichment of intracellular free l-[3H]phenylalanine and the enrichment in plasma at early time points (10 min), although at both amounts, the enrichment in the two compartments had equalized by 30 min (38).

At 45 min, the enrichment of phenylalanine in plasma was only ~58% of that of the injected solution in dogs, compared with ~75% in human volunteers (38), indicating faster clearance of phenylalanine in dogs. Because the rate of muscle protein synthesis is only 20–30% higher in the dog than in humans (see summary in Ref. 19), the more rapid decline of plasma phenylalanine enrichment may be explained by both more rapid whole body protein synthesis and higher amino acid oxidation rates in dogs compared with humans (e.g., Refs. 6, 14, 24, 29, and 30).

An even larger flooding dose, such as 248 mg/kg, generally used in rodents (20) or other animals (11, 45), would have maintained higher levels of plasma phenylalanine enrichment for a longer time, as well as facilitating equilibration of the enrichment of phenylalanyl-tRNA and plasma phenylalanine.

The value of muscle protein synthesis for the basal period assessed with the flooding technique (Table 2) is only ~50% of the rate reported by Jahoor et al. (31), with a flooding amount of l-[13C]leucine in dogs (5.0 ± 0.43%/day). It is possible that this difference arises from a stimulation of muscle protein synthesis by the bolus injection of leucine, as has been suggested from studies in other systems both in vitro (7, 34, 46) and in vivo (7, 44). With phenylalanine, however, the injection of a large amount of amino acid did not stimulate the rate of muscle protein synthesis, as can be seen from the similarity of rates obtained with the bolus injection of a large amount of phenylalanine and those obtained with tracer amounts of labeled phenylalanine given as a constant infusion (Table 2 and Ref. 9).

There is a remarkable similarity in the rates of muscle protein synthesis obtained with the flooding technique and the constant infusion technique (9) calculated with phenylalanine enrichment in either tissue fluid or phenylalanyl-tRNA (Table 2). There are no significant differences in the basal rate of protein synthesis among any of the measurements, excluding those from constant infusion based on plasma phenylalanine enrichment (ANOVA). Likewise, there are no significant differences among the rates of muscle protein synthesis during the infusion of insulin with amino acids for any of the three possible precursors with the flooding technique (experiments 1 and 2) and the constant infusion technique based on phenylalanine enrichment in either tissue fluid or phenylalanyl-tRNA (Table 2). However, the similarity of rates of muscle protein synthesis assessed with constant infusion and the flooding technique in this study has not been consistently observed in human studies. In some studies in humans, rates of muscle protein synthesis from constant infusion were lower (e.g., Refs. 4 and 43) than those assessed with the flooding technique (e.g., Refs. 21 and 38). However, other studies in humans have reported rates from constant infusion that were similar to rates reported with the flooding technique (1, 5, 47).

This study has also explored the relationship of the enrichment of leucyl-tRNA to plasma KIC, often used as a surrogate for the enrichment of leucine at the site of protein synthesis. The enrichment of leucine in tissue fluid was similar to that of leucyl-tRNA, both in the
basal state (leucyl-tRNA-to-tissue fluid enrichment 0.95 ± 0.02) and during the infusion of insulin and amino acids (leucyl-tRNA-to-tissue fluid enrichment 0.94 ± 0.02, Fig. 7A). However, the enrichment of KIC in plasma was significantly higher than the enrichment of leucyl-tRNA (27 ± 8% higher in the basal state, P = 0.01, and 10 ± 4% higher with insulin and amino acids, P = 0.02). A similar relationship of the enrichment of leucyl-tRNA to that of plasma KIC was also observed when a tracer amount of leucine was infused without a continuous infusion with a flooding amount of phenylalanine (KIC enrichment 29 ± 4% higher during the basal state, P = 0.0002; and 21 ± 5% higher during the infusion of insulin and amino acids, P = 0.002) (unpublished data from Ref. 9). These data suggest a compartmentation of intracellular leucine pools, with a greater proportion of leucine from plasma going into the pool supplying the oxidative pathway (via KIC) than into the pool supplying protein synthesis (leucyl-tRNA). This compartmentation is most evident in the basal state. With provision of insulin and amino acids, the intracellular pool of leucine becomes more homogeneous.

Although the present study in the adult dog demonstrates a stimulation of muscle protein synthesis by insulin with amino acids, many studies in healthy human subjects have observed no stimulation of muscle protein synthesis by insulin when amino acid levels were allowed to decline (38, 41) or when amino acid levels were maintained at basal levels (16, 22, 26, 36). However, a few studies in adult volunteers have reported a stimulation of muscle protein synthesis with insulin infusion at physiological (4) and supraphysiological levels (28). While concomitant infusion of exogenous amino acids with insulin provides substrate amino acids for protein synthesis, there is also a potential for these amino acids to alter the relationship of aminoacyl-tRNA enrichment relative to the enrichment of the tracee in plasma (9). The value of the flooding technique is that there is less potential for alterations in the enrichment of the precursor, as demonstrated by the close agreement of plasma phenylalanine enrichment and phenylalanyl-tRNA even with the infusion of insulin and amino acids.

The present study in dogs does not explain the difference in response of muscle protein synthesis to insulin between adult dogs and humans in several of the human studies. It is possible that there is a genuine difference in response between these two species, but it is also possible that the stage of development is different between the adult human and 20-kg dogs. The sensitivity of muscle protein to the stimulatory effect of insulin is age related, with stimulation by insulin declining during development in both rats and pigs (2, 48). At 20 kg, it is possible that dogs might be sufficiently immature to maintain responsiveness to stimulation by insulin comparable to that of other growing animals.

In summary, this study shows that infusion of insulin along with amino acids in dogs stimulates the rate of muscle protein synthesis. The study also documents that the enrichments of plasma free phenylalanine and phenylalanyl-tRNA were comparable at the end of a 45-min incorporation period. The rates of muscle protein synthesis calculated from plasma phenylalanine enrichment were comparable to those obtained with phenylalanyl-tRNA both in the basal state and during infusion of insulin and amino acids, and also to values obtained with the constant infusion of tracer amounts of phenylalanine. Thus, by minimizing the differences in the enrichment among all amino acid precursor pools, the flooding technique allows an accurate estimate of muscle protein synthesis with sampling of the more accessible plasma amino acid pool, obviating the necessity of assessing the enrichment of aminoacyl-tRNA.

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