Quantification of amino acid transport through interstitial fluid: assessment of four-compartment modeling for muscle protein kinetics

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Gore DC, Wolfe RR, Chinkes DL. Quantification of amino acid transport through interstitial fluid: assessment of four-compartment modeling for muscle protein kinetics. Am J Physiol Endocrinol Metab 292: E319–E323, 2007. First published September 5, 2006; doi:10.1152/ajpendo.00399.2005.—The purpose of this study was to assess a novel technique for quantifying in vivo muscle protein metabolism and phenylalanine transport in septic patients and normal volunteers and thereby assess the influence of sepsis on muscle protein kinetics. In patients resuscitated from sepsis, blood flow and edema may influence the extent of muscle loss. Six adult patients septic from pneumonia underwent a study protocol consisting of infusion of isotopic phenylalanine, indocyanine green dye, and sodium bromide; biopsies of skeletal muscle; and sampling from the femoral artery, vein, and interstitial fluid. Study results demonstrate a substantial net catabolism of muscle, an accelerated flux of phenylalanine, and an increased leg blood flow for septic patients compared with normal volunteers. For septic patients and normal volunteers, the rate of phenylalanine transport through the interstitium was rate limiting for the movement of phenylalanine between vasculature and muscle. Measurements demonstrate a concentration gradient of phenylalanine favoring the net efflux of amino acids from the leg in the septic patients. Despite whole body edema, the extracellular fluid volume within muscle of septic patients was similar to normal. These findings demonstrate that the extent of muscle loss in critically ill patients results from the net increase in the rate of muscle protein breakdown, which subsequently drives amino acids through the interstitial compartment down their concentration gradient. Therefore, any effective therapy to correct illness-induced muscle catabolism should be directed at altering the rates of breakdown and synthesis of muscle protein and are not likely related to tissue edema.

 MATERIALS AND METHODS

Study subjects. The study protocol was completed in eight subjects. All study subjects were septic, with a minimal sepsis severity score of 14. Furthermore, all subjects had pneumonia as an infectious source of their sepsis, as evidenced by lobar consolidation on chest radiographs and bacterial organisms identified on culture of either bronchoscopic lavage or undirected aspiration via the endotracheal tube. At the time of study, all subjects were receiving enteral nutrition delivered continuously via a nasojejunal tube. All subjects had good hemodynamics and adequate urine output. No subject had overt renal (serum creatine ≥1.5 mg/dl) or liver dysfunction (bilirubin ≥1.135 mg/dl). Only two study subjects had a pH ≤7.30, lactate ≥5 mmol/l). Patients received fentanyl for analgesia and midazolam for sedation. No subject was chemically paralyzed prior to or during study.

Of the eight patients who completed the metabolic assessment, two subjects were subsequently excluded because the isotopic enrichment of fluid sampled from the interstitial space was exceedingly low. After study completion, blood clots were evident on the microdialysis catheters on both of these subjects, thus strongly suggesting catheter occlusion as a probable explanation for the very low isotopic enrichment of the effluent. The characteristics of the remaining six subjects presented in Table 1. These subjects ranged in age from 31 to 61 yr and in weight from 56 to 96 kg. Four of these six subjects required mechanical ventilatory support at the time of study. As predisposing factors for the sepsis pneumonia, three patients had suffered recent burn injuries and three subjects had suffered extensive traumatic injuries. Two study subjects subsequently died: one following an acute myocardial infarction 2 days following study; the other patient succumbed to progression of sepsis to multiorgan failure and died 29 days following study.

Regardless of the extent of nutritional support, critically ill patients pervasively lose muscle mass (12). With prolonged illness, this loss of muscle adversely affects immune function, wound healing, and eventual overall survival (15). In an effort to combat this net catabolism of muscle, our group and others have investigated a variety of nutritional regimens as well as various anabolic agents (17). For example, such agents as insulin, growth hormone, testosterone, and others have been shown to augment the rate of muscle protein synthesis and thereby reduce and even negate the net loss of muscle in severely injured and critically ill patients (4–6). Stable isotope tracer methodology has for several years been the primary means for assessing in vivo the efficacy of these various regimens on muscle protein kinetics (1). By combining serial arterial and venous blood sampling, muscle biopsies, and a measure of leg blood flow, quantification of not only the rates of muscle protein synthesis and breakdown but also the rates of amino acid transport into and out of muscle can be determined. Unfortunately, this three-pool compartment model (artery, vein, muscle) fails to assess the influence of the interstitial fluid space on muscle protein kinetics. Given the fact that patients resuscitated from severe injury and sepsis are exorbitantly edematous, it is quite plausible that the changes in the interstitial compartment associated with critical illness may have a demonstrative impact on the movement of amino acids into and out of muscle. The purpose of this study was to assess a novel technique for quantifying not only the kinetics of muscle protein synthesis and breakdown but also the rates of amino acid transport into and out of the interstitial space in critically ill patients compared with healthy normal subjects. It is hoped that, by employing this four-compartment modeling methodology, a comprehensive assessment of injury-induced muscle catabolism, specifically the influence of the interstitial space and extremity blood flow, can be determined.

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were obtained from the vastus lateralis muscle by means of a Berg-QDR-4500A, Waltham, MA). With local anesthesia, muscle biopsies were performed and muscle biopsies were analyzed for both intracellular and protein-bound amino acid concentration and isotopic enrichment by use of an internal standard method of analysis by GC-MS (3). To ~20 mg of muscle were added 800 µl of 14% perchloric acid and 2 µl of internal standard. Samples were homogenized and centrifuged, and the supernatant was collected. This procedure was repeated twice more and the pooled supernatant processed identically to the blood samples as described above using tert-butyldimethylsilyl derivatization and analysis by GC-MS. This method determined the free intracellular concentration and isotopic enrichment for phenylalanine. For determination of protein-bound concentration and enrichment, the remaining muscle pellet was washed repeatedly with saline and absolute ethanol, dried, and then hydrolyzed with 6 N HCl. The protein hydrolysate was then passed over a cation exchange column (Dowex AG; Bio-Rad Laboratories, Richmond, CA), dried, esterified, heated, and subsequently analyzed by GC-MS using chemical impact ionization as previously described (16).

Calculations. The net balance of phenylalanine across the leg was calculated as follows and expressed per 100 ml leg volume:

\[ AVNB = (C_a - C_c) \times LBF \]

where AVNB is net balance of phenylalanine across the leg (nmol·min⁻¹·100 ml leg volume⁻¹); Ca, Cc is concentration of phenylalanine from artery and vein, respectively (nmol/ml); and LBF is leg blood flow (ml·min⁻¹·100 ml leg volume⁻¹). Thus a negative net balance reflects the net release of phenylalanine from the limb.

Isotopic steady state in the free amino acid pools of blood, interstitium, and muscle is required for the four-compartment modeling

Table 1. Patient characteristics

<table>
<thead>
<tr>
<th>Normal Volunteers (n = 6)</th>
<th>Septic Patients (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yr</td>
<td>27±8</td>
</tr>
<tr>
<td>Body weight, kg</td>
<td>84±6</td>
</tr>
<tr>
<td>Sepsis severity score</td>
<td>0</td>
</tr>
<tr>
<td>APACHE II score</td>
<td>0</td>
</tr>
<tr>
<td>Mean BP, mmHg</td>
<td>84±4</td>
</tr>
<tr>
<td>PaO2/FiO2</td>
<td>0</td>
</tr>
<tr>
<td>Serum creatinine, mg/dl</td>
<td>0.4±0.1</td>
</tr>
<tr>
<td>Serum bilirubin, mg/dl</td>
<td>0</td>
</tr>
</tbody>
</table>

Values are means ± SD. BP, blood pressure.

Study protocol. This study was approved by the Institutional Review Board of The University of Texas Medical Branch, Galveston, TX, and consent was obtained from family. The study protocol is diagramed in Fig. 1 and was performed in each patient’s intensive care unit room. Local anesthesia (1% lidocaine) facilitated placement of vascular access catheters into an adjacent femoral artery and vein as well as insertion of the microdialysis probes (mol mass <=3,000 Da) into the vastus lateralis muscle, as described previously (8).

After baseline blood sampling, a primed continuous infusion of [14C]phenylalanine (2 µmol/kg prime; 0.07 µmol·kg⁻¹·min⁻¹) was given via central venous access and continued for the 5 h of study. The microdialysis probes were perfused with Ringer’s lactate solution at a rate of 5 µl/min. A radioactive tracer of phenylalanine was added to the perfusate to quantify the recovery rate of this amino acid from the interstitial fluid (8). Leg blood flow was determined by infusion of indocyanine green dye (1 mg/min per 20 min) into the femoral artery with subsequent spectrophotometric analysis of blood drawn simultaneously from the femoral vein and from the central vein access (7). Leg blood flow measurements were standardized for leg volume as assessed by integrating several circumference measurements with the length of the calf, thigh, and foot. Extracellular fluid volume was determined by infusion of 3% NaBr solution at a dose rate of 0.75 ml/kg body wt into the femoral artery for 30 min (10). Subsequent spectrophotometric analysis of blood drawn sequentially from the central venous access allowed quantification of whole body extracellular fluid volume. Blood drawn sequentially from the femoral venous access was standardized for the leg muscle mass as subsequently quantified by dual-energy X-ray absorptiometry (DEXA; Hologic-QDR-4500A, Waltham, MA). With local anesthesia, muscle biopsies were obtained from the vastus lateralis muscle by means of a Bergstrom needle. Muscle biopsies were performed at the second and fifth hours of study. Dialysate fluid from the microdialysis probes were collected for subsequent analysis from the fourth to fifth hours of study. After 4.5, 4.75, and 5 h of isotopic infusion, blood was drawn simultaneously from the femoral artery and vein, thus completing the baseline metabolic measurements. Following completion of the study protocol, each patient underwent DEXA, thereby quantifying muscle mass within the studied leg.

Sample analysis. Isotopic enrichment of phenylalanine and the blood and dialysate concentration of unlabeled phenylalanine were determined by gas chromatography-mass spectrometry (GC-MS), using an internal standard method (16). Blood and dialysate were collected in ice-cold tubes containing 2 ml of 15% sulfosalicylic acid and 200 µl of internal standard ([13C]phenylalanine). Samples were vortexed and centrifuged. The supernatant was then frozen at −80°C until processing. After tert-butyldimethylsilyl derivatization, plasma samples were then analyzed with GC-MS (model 5989; Hewlett-Packard, Palo Alto, CA) using electron impact ionization.

Muscle biopsies were analyzed for both intracellular and protein-bound amino acid concentration and isotopic enrichment by use of an internal standard method of analysis by GC-MS (3). To ~20 mg of muscle were added 800 µl of 14% perchloric acid and 2 µl of internal standard. Samples were homogenized and centrifuged, and the supernatant was collected. This procedure was repeated twice more and the pooled supernatant processed identically to the blood samples as described above using tert-butyldimethylsilyl derivatization with analysis by GC-MS. This method determined the free intracellular concentration and isotopic enrichment for phenylalanine. For determination of protein-bound concentration and enrichment, the remaining muscle pellet was washed repeatedly with saline and absolute ethanol, dried, and then hydrolyzed with 6 N HCl. The protein hydrolysate was then passed over a cation exchange column (Dowex AG; Bio-Rad Laboratories, Richmond, CA), dried, esterified, heated, and subsequently analyzed by GC-MS using chemical impact ionization as previously described (16).

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Study Protocol Flow Diagram

Fig. 1. Study protocol flow diagram. ICG, indocyanine green.
calculations. Isotope phenylalanine was infused for 300 min to achieve isotopic equilibrium, which was evidenced by the steady enrichment in the serial blood sampling during the final 30 min of the study protocol. The model is shown schematically in Fig. 2 and provides quantification of amino acid transport into and out of muscle as well as the rates of muscle protein synthesis and breakdown (9). Values are calculated as follows and all are expressed as nanomoles per minute per 100 milliliters of leg volume:

\[
F_{in} = C_v \times LBF \\
F_{out} = C_v \times LBF \\
F_{va} = F_{out} - F_{vi} \\
F_{vi} = \left(\frac{(E_v - E_i)}{(E_v - E_a)}\right) \times C_v \times LBF \\
F_{ma} = F_a - F_{va} \\
F_{mi} = F_{ma} + \left(\frac{(E_m - E_i)}{(E_m - E_a)}\right) \times C_v \times LBF \\
F_{mv} = F_{mi} + (F_{in} - F_{out}) \\
F_{mo} = F_{mv} \times (E_v/E_m - 1) \\
F_{im} = F_{mo} + AVNB \\
R_{in} = F_{ma} + F_{mv} \\
\]

where \(F_a\) is flow of amino acid into leg, \(F_{out}\) is flow of amino acid out of leg, \(F_{ma}\) is flow of amino acid from artery into muscle, \(F_{vi}\) is flow of amino acid from interstitial space to vein, \(F_{mv}\) is flow of amino acid from artery to interstitial space, \(F_{im}\) is flow of amino acid from muscle to interstitial space, \(F_{mo}\) is flow of amino acid from interstitial space to vein, \(F_{mv}\) is flow of amino acid from artery to interstitial space, \(F_{im}\) is flow of amino acid from muscle to interstitial space, \(R_{in}\) is total rate of intracellular appearance of phenylalanine; \(E_m\), \(E_v\), \(E_i\), and \(E_m\) are isotopic enrichment of phenylalanine in arterial plasma, venous plasma, interstitial fluid, and muscle sample, respectively [molar percent excess (MPE)].

Because phenylalanine is an essential amino acid in which production from the leg can come only from breakdown of muscle protein, \(F_{mo}\) for phenylalanine is a direct reflection of the rate of muscle protein breakdown. Likewise, the disappearance of phenylalanine (\(F_{om}\)) reflects the rate of muscle protein synthesis.

To index the efficiency of transmembrane transport accounting for changes in leg blood flow and alterations in amino acid availability, the rates of amino acid transport were normalized by the rate of amino acid delivery into a given compartment and expressed as follows:

\[
efficiency F_{ia}(\%) = \frac{F_{ia}}{F_{in}} \\
efficiency F_{im}(\%) = \frac{F_{im}}{F_{ma}} \\
efficiency F_{mo}(\%) = \frac{F_{mo}}{F_{mi}} \\
efficiency F_{iv}(\%) = \frac{F_{iv}}{F_{mv}} \\
\]

The concentrations of phenylalanine in femoral arterial and venous blood samples and in muscle (\(C_m\)) were measured directly. The phenylalanine concentration in the interstitium (\(C_i\)) was calculated as follows:

\[
C_i = (C_v - C_m(1 - (F_{im}/F_{mi}))) / (F_{im}/F_{mi}) \\
\]

Statistical analysis. A Student’s t-test was used to assess statistical significance at \(P \leq 0.05\).

RESULTS

Measurements in critically ill septic patients are compared with values for normal volunteers that have been reported previously and obtained using identical methodology (9). Characteristics of these septic patients and normal volunteers are noted in Table 1. Leg blood flow and the net balance of phenylalanine across the leg are noted in Table 2. The negative net balance of \(-92 \text{ mmol} \cdot \text{min}^{-1} \cdot 100 \text{ ml leg volume}^{-1}\) is indicative of substantial net muscle catabolism. Calculated values for the rate of phenylalanine transport into the leg and the net flow of phenylalanine out of the leg are shown also in Table 2. These values are significantly higher than those evident for normal volunteers, thus indicating the greater flux of amino acids into and out of muscle for these septic patients. Furthermore, the fractional synthetic rate of muscle protein as shown in Table 2 is substantially greater than reported values for normals (11), another indication of a greater flux of muscle protein in these septic, hypermetabolic patients. Calculated values for muscle protein breakdown (\(F_{mo}\)) and muscle protein synthesis (\(F_{om}\)) are reported in Table 2. These values are substantially greater than those reported for normal volunteers, further indexing the greater net catabolism and faster turnover of phenylalanine within muscle protein for septic patients.

The rates of phenylalanine transport between compartments are shown in Fig. 3. The rate of transport of phenylalanine from artery to muscle in septic patients is similar to that evident in normal volunteers. Furthermore, the rate of flow of phenylalanine from artery into the interstitial compartment and from the interstitial compartment into muscle is also similar between critically ill patients and normal volunteers. In contrast, the flow of phenylalanine from muscle to interstitium and from
interstitium to vein was nearly 20 and 50% higher, respectively, for critically ill patients compared with healthy normal subjects, thereby demonstrating an accelerated transport of amino acid out of muscle and into the venous circulation. The shunt of phenylalanine directly from artery to vein for critically ill patients was more than double that evident for normal volunteers.

The efficiency of transport between tissue compartments is noted in Table 3. Values for septic patients were similar to those of normal volunteers for the rate of transport of phenylalanine from interstitium to muscle, from muscle to interstitium, and from interstitium to vein. The efficiency of transport of phenylalanine from the artery to interstitium was >35% less in the septic patients compared with the normal volunteers. As shown in Table 4, concentrations of unbound phenylalanine were highest in muscle, less for interstitium and artery, and lowest in the venous sample. Compared with normal volunteers, absolute concentrations were higher in septic patients for each compartment except those from the femoral vein.

Extracellular fluid volume was quantified by NaBr dilution and is shown in Table 5. For the critically ill subjects, whole body extracellular water measured 24 liters. When indexed by body weight, these septic patients evidenced an extracellular fluid volume of 30% of their total body mass. For the leg of body weight, these septic patients evidenced an extracellular water measured 1.6 liters. When indexed by the lean body mass of the leg as quantified by DEXA, septic patients evidenced an extracellular fluid volume within leg muscle of 18%.

Table 3. Efficiencies of phenylalanine transport between compartments

<table>
<thead>
<tr>
<th>Compartment</th>
<th>Normal Volunteers</th>
<th>Critically Ill Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Artery → Interstitium (F_{im} %)</td>
<td>56 ± 3</td>
<td>34 ± 4*</td>
</tr>
<tr>
<td>Interstitium → Muscle (F_{mi} %)</td>
<td>68 ± 3</td>
<td>60 ± 6</td>
</tr>
<tr>
<td>Muscle → Interstitium (F_{mi} %)</td>
<td>82 ± 8</td>
<td>73 ± 8</td>
</tr>
<tr>
<td>Interstitium → Vein (F_{vi} %)</td>
<td>31 ± 4</td>
<td>40 ± 5</td>
</tr>
</tbody>
</table>

Values are means ± SD. See Calculations for definitions. *P < 0.05.

Table 4. Phenylalanine concentrations within each compartment

<table>
<thead>
<tr>
<th>Compartment</th>
<th>Normal Volunteers</th>
<th>Septic Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>C_s</td>
<td>66 ± 1</td>
<td>78 ± 4*</td>
</tr>
<tr>
<td>C_c</td>
<td>71 ± 2</td>
<td>96 ± 5</td>
</tr>
<tr>
<td>C_l</td>
<td>77 ± 2</td>
<td>131 ± 14*</td>
</tr>
<tr>
<td>C_{ve}</td>
<td>90 ± 4</td>
<td>146 ± 14*</td>
</tr>
</tbody>
</table>

Values are means ± SD in nmol/ml. *P < 0.05.

DISCUSSION

Previous application of this four-compartment study methodology to normal healthy volunteers demonstrated that interstitial fluid represents a distinct compartment in the movement of amino acids into and out of muscle, with the flow across the interstitium much slower than the rate of amino acid transport into muscle (9). This finding supported the concept and prior in vitro evidence of an active transport of amino acids into muscle (14). In contrast, the transient of amino acids across the interstitium appears largely dependent on diffusion and thereby rate limiting for the movement of amino acids from the vascular system and into and out of muscle. These results have a significant implication to critically ill and severely injured patients in which alterations in extremity blood flow and exaggerated increases in peripheral edema would be anticipated to have a substantial influence on muscle protein kinetics and possibly play a key role in the extent of muscle catabolism. In the present study involving modestly septic patients with pneumonia, the absolute rates of phenylalanine transport from artery to interstitium and from interstitium to vein were substantially less than rates of transport into and out of muscle. Thus, in critically ill patients, as in normal healthy subjects, the interstitial compartment functionally limits the rate of amino acid movement between vasculature and muscle. Therefore, while transport mechanisms along the muscle cell membrane actively uptake amino acids into muscle, the delivery of these amino acids to the membrane surface are limited by diffusion through the interstitial fluid space.

On the basis of results from the study of normal volunteers, we theorized that illness-related edema would substantially reduce the rates of amino acid transport through an expanded interstitial fluid compartment. Using the Fick dilution principle, we used NaBr to measure the interstitial fluid compartment in these modestly septic patients. This technique quantified total body extracellular fluid volume as nearly 30% of the body mass, a value 50% greater than the 20% extracellular fluid volume considered normal for a 70-kg man (18). This is consistent with the clinical impression of substantial whole body edema in these patients. However, when the NaBr dilu-
ion values were indexed by the lean body mass of the leg, the interstitial fluid compartment for muscle measured only 18%. These measurements suggest that, in contrast to the expanded extracellular fluid volume for the entire body, actual edema within the muscle of severely ill patients is minimal. One possibility to explain this discrepancy between extensive whole body edema with yet only a modest increase in the interstitial fluid volume within muscle may be related to the limits of expansion confined by the fascia that surrounds extremity muscles. Thus, despite the septic inflammatory stimulus for edema formation, the interstitial fluid compartment of muscle in these critically ill patients remains similar to the assumed value for the normal healthy subjects. This similarity in interstitial fluid volume may be a significant factor in the observed similarity in transport rates of phenylalanine through the interstitium for both septic patients and healthy volunteers despite the substantial differences in the flow of phenylalanine into and out of the muscle and the rates of muscle protein synthesis and breakdown. Unfortunately, the similarity in rates of amino acid transport through the interstitium between critically ill patients and normal subjects, combined with their similarity in the extracellular fluid volume for muscle, negates our ability to assess any association between extracellular fluid volume and amino acid transport through the interstitial compartment.

Prior kinetic studies have suggested that circulatory changes in subjects following resistance exercise (2) or in patients following a severe injury (13) result in an increased delivery of amino acids into the leg, which then impacts the exchange of amino acids between blood and muscle. By use of four-compartment modeling methodology, this study demonstrated that, despite an increase in leg blood flow and a nearly 40% increase in delivery of phenylalanine into the leg, there was no overt change in the rate at which this amino acid transverses the interstitium, thereby disproving the contention that blood flow has a major impact on the amino acid transport into and out of muscle. This observation supports further the notion that amino acid transport through the interstitial fluid compartment is rate limiting for the movement of amino acids into and out of muscle. Furthermore, when the rates of phenylalanine transport into and out of each compartment are indexed as a coefficient of transport, this study demonstrated that the efficiency of amino acid transport across the muscle cell membrane remains similar between critically ill patients and normal volunteers. In contrast, the efficiency of transport from the interstitial fluid to the vein is increased slightly with critical illness. Conversely, the transport efficiency from artery to interstitial fluid is decreased in critically ill patients. Measures of the concentration of phenylalanine within each of the compartments demonstrate that the concentration gradient is increased in septic patients, favoring outward transport of amino acids from muscle, a finding which may explain the alterations in transport efficiency between artery through interstitial fluid and interstitial fluid to vein. Undoubtedly, a major factor affecting these changes in concentration within each phenylalanine compartment is the accelerated rate of protein breakdown in excess of the rate of muscle protein synthesis. Results suggest that the increased availability of phenylalanine and other amino acids from the accelerated rate of protein breakdown drives the amino acids out of the muscle and through the interstitium down their concentration gradient. Therefore, it appears that the efflux of amino acids from muscle into the vasculature is primarily related to alterations in the rate of net muscle protein catabolism and is not likely related to any alterations in tissue edema or muscle blood flow. In the context of these results, any effective therapy designed to correct or negate illness-induced muscle catabolism should be focused at altering the rates of protein breakdown and synthesis in muscle. Conversely, any attempt to negate tissue edema or normalize extremity blood flow is not likely to be of any benefit in countering muscle loss. Furthermore, given the passive nature of amino acid diffusion through the interstitium, measures from the interstitial, fourth compartment do not add or alter findings as calculated from a traditional three-compartment study. In view of the greater complexity and invasive nature, this fourth-compartment methodology appears unwarranted.

GRANTS

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REFERENCES