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Method for the determination of the arteriovenous muscle protein balance during non-steady-state blood and muscle amino acid concentrations

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Katsanos, Christos S., David L. Chinkes, Melinda Sheffield-Moore, Asle Aarsland, Hisamine Kobayashi, and Robert R. Wolfe. Method for the determination of the arteriovenous muscle protein balance during non-steady-state blood and muscle amino acid concentrations. *Am J Physiol Endocrinol Metab* 289: E1064–E1070, 2005. First published August 9, 2005; doi:10.1152/ajpendo.00141.2005.—We describe a method based on the traditional arteriovenous balance technique in conjunction with muscle biopsies for the determination of leg muscle protein balance during the nonsteady state in blood amino acid concentrations. Six young, healthy individuals were studied in the postabsorptive state (pre-Phe) and after a bolus ingestion of ~0.5 g phenylalanine (post-Phe). Post-Phe free phenylalanine concentrations in blood and muscle increased ($P < 0.05$), but the respective concentrations of the amino acid threonine did not change. The average post-Phe leg net balance (NB) for threonine decreased from basal ($P < 0.05$), but that for phenylalanine did not change. A volume of distribution for free phenylalanine in the leg was calculated based on the leg lean mass and the relative muscle water content and used to estimate the rate of accumulation of free phenylalanine in the leg. When the post-Phe NB for phenylalanine was corrected for the rate of accumulation of free phenylalanine in the leg, the post-Phe NB for phenylalanine decreased from basal ($P < 0.05$). This corrected value was not different ($P > 0.05$) from the value predicted for the phenylalanine NB based on the pre- and post-Phe NB responses for threonine. We conclude that the protein NB in non-steady-state blood phenylalanine concentrations can be determined from the arteriovenous phenylalanine NB by accounting for changes in free phenylalanine within its volume of distribution.

methodology; phenylalanine net balance; intracellular phenylalanine; volume of distribution

THE ARTERIOVENOUS NET BALANCE (NB) technique has been used extensively in humans to evaluate rates of protein synthesis and breakdown in forearm and leg muscles (1, 3, 7, 9, 15, 25, 29, 31, 33) as well as other tissues (17) under a variety of physiological conditions. In the above studies, as well as others (18, 28, 30, 32), the amino acid phenylalanine has been used to trace muscle protein balance. Phenylalanine was chosen because it is neither produced nor metabolized in muscle, thereby enabling extrapolation from phenylalanine NB to muscle protein NB. Previous research has validated the use of the leg arteriovenous NB technique to study muscle protein kinetics during a steady state in the concentration of blood amino acids (2, 4).

A general problem with the use of either the forearm or leg arteriovenous NB technique to evaluate muscle metabolism in

a non-steady-state situation, such as after the ingestion of a meal or a bolus of protein or amino acids, is that the transit time of the substance of interest through the forearm or the leg increases as the arterial concentration of the substance increases due to the temporal accumulation of that substance in extravascular tissues (24). The increase in the arteriovenous transient time of the substance of interest (e.g., phenylalanine) makes the interpretation of muscle protein balance data collected while the blood concentration of that substance is changing (i.e., nonsteady state) difficult to interpret.

The problem associated with the determination of muscle protein NB in a non-steady-state situation can be addressed by collecting frequent blood samples throughout the nonsteady state and until the concentration of free phenylalanine in the plasma and the extravascular tissues returns to its basal value. In that case, reliable data for muscle protein NB can be obtained from phenylalanine NB, provided that an integrated value for the entire arteriovenous phenylalanine NB response is calculated. However, this approach has practical limitations associated with the time and the blood required and, more importantly, does not permit the evaluation of short-lived effects on muscle protein metabolism. Knowledge of the volume of distribution of free phenylalanine in the extravascular tissues allows accounting for the phenylalanine NB associated with changes in free phenylalanine within its volume of distribution. Previous reports have made assumptions (e.g., fixed %leg volume) regarding the volume of distribution of free phenylalanine in the leg (25, 31), but these assumptions have never been validated.

The purpose of this study was to evaluate a method to determine muscle protein balance based on the leg arteriovenous phenylalanine NB technique during a nonsteady state in blood and muscle free phenylalanine concentrations. We hypothesized that, when account is taken of the change in the free phenylalanine concentration within its volume of distribution, which can be estimated from measures of leg lean mass and relative muscle water content, valid measures of muscle protein balance can be obtained.

MATERIALS AND METHODS

Subjects

Seven healthy subjects (3 females and 4 males) with age 24 ± 3 yr (mean \pm SE), body mass 76 ± 4 kg, height 172 ± 4 cm, and body fat

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24 ± 4%, took part in the study. Leg volume (12.0 ± 0.4 liters) was determined using an anthropometric method. This method is based on partitioning the leg into six segments, which are viewed as truncated cones, and the foot, which is viewed as a wedge (19, 22). Leg lean mass (9.4 ± 0.6 kg) was determined using dual-energy X-ray absorptiometry (DEXA).

Subjects were determined to be healthy based on medical history, physical examination, resting electrocardiogram, and routine blood and urine tests. The purpose, design, and the risks associated with this study were explained to each subject before written consent was obtained. The study protocol was approved by the Institutional Review Board and the General Clinical Research Center (GCRC) of the University of Texas Medical Branch at Galveston.

Isotopes

L-[ring-¹³C₆]phenylalanine (99% enriched), L-[U-¹³C₉,¹⁵N]phenylalanine (¹³C₉ 98% enriched; ¹⁵N 98% enriched), L-[U-¹³C₄,¹⁵N]threonine (U-¹³C₄ 98% enriched; ¹⁵N 98% enriched), and L-[¹⁵N]threonine (98% enriched) were purchased from Cambridge Isotope Laboratories (Andover, MA). Isotopes were confirmed sterile and pyrogen free by the manufacturer. Tracers were dissolved in normal saline (0.9% NaCl) the night before the experiment.

Placement of the Catheters

Subjects were admitted to the GCRC the evening before the study. They ate dinner and consumed nothing after 10:00 PM. The next morning (~5:00 AM), an 18-gauge polyethylene catheter was inserted in an antecubital vein of each arm. One catheter was used for infusion of the labeled amino acids, and the other catheter in the opposite arm was used for collection of blood to determine recycling of dye to calculate leg blood flow using the dye-dilution technique. At ~7:00 AM, 3-Fr, 8-cm polyethylene catheters (Cook, Bloomington, IN) were inserted in the femoral artery and vein of one leg under local anesthesia and used for arteriovenous blood sampling across the leg. Patency of all catheters was maintained by saline (heparinized saline for the femoral catheters) infusion.

Experimental Protocol

The experimental protocol was composed of the following two phases: the prephenylalanine ingestion period from -2 to 0 h and the postphenylalanine ingestion period, which was followed for up to 5 h after the phenylalanine ingestion (3 subjects were studied for 4 h and 4 subjects for 5 h). Infusion of the tracers was started after the collection of a blood sample for the measurement of background amino acid enrichments and indocyanine green (ICG) concentration (~7:00 AM), and the infusion of the tracers was maintained constant until the end of the experiment. The tracers used, as well as the infusion rates (IR) and priming doses (PD), were as follows: L-[ring-¹³C₆]phenylalanine, IR: 0.05 μmol·kg⁻¹·min⁻¹; PD: 2.0 μmol/kg; L-[U-¹³C₄,¹⁵N]threonine, IR: 0.08 μmol·kg⁻¹·min⁻¹; PD: 7.8 μmol/kg. ICG dye (Akorn, Buffalo Grove, IL) infusion was started at a constant rate (0.5 mg/min) into the femoral artery at two different times during the experiment (-1 and +2 h) and maintained for ~15 min each time to determine leg blood flow.

Blood samples for the determination of amino acid enrichments and concentrations were drawn from the femoral artery and venous catheters at -60, -40, -30, -20, and -5 min before phenylalanine ingestion. At 0 h (~9:00 AM), a drink was ingested that contained 0.508 g phenylalanine dissolved in 250 ml of a noncaloric soft drink. L-[ring-¹³C₆]phenylalanine (0.047 g) was added to the drink to maintain the isotopic enrichment of phenylalanine at a steady state (~9%) during the postphenylalanine ingestion period. Blood samples were drawn every 15 min after the phenylalanine ingestion until the end of the experiment. Blood samples for the determination of leg blood flow were collected simultaneously from the femoral and peripheral veins.

For that purpose, four sets of blood samples were collected at least 10 min after the start of each ICG infusion.

Three muscle biopsies were performed during the experiment. The first biopsy was performed just before the phenylalanine ingestion, the second one was performed 1 h after the phenylalanine ingestion, and the third one was collected at the end of the study. The muscle biopsies were taken on the lateral portion of vastus lateralis (~15–20 cm above the knee) from 7- to 8-mm incisions using sterile technique after anesthetizing the skin and the subcutaneous tissue with 1% lidocaine. The first two muscle biopsies were taken from the distal incision and the third one from the proximal incision, using a 5-mm Bergstrom biopsy needle (Depuy, Warsaw, IN). The needle was advanced through the skin and fascia deep in the muscle with the cutting edge of the needle closed. While suction was applied, the cutting cylinder was opened and closed three times. About 50 mg muscle tissue were obtained with each biopsy, which, after removing any visible fat and connective tissue, was rinsed with ice-cold saline to remove blood and then blotted dry. The sample, which was composed of mixed fibers of skeletal muscle, was immediately frozen in liquid nitrogen and stored at -80°C until analysis.

Sample Analyses

Blood. Blood collected from the femoral artery and vein was used for the determination of the phenylalanine and threonine enrichments and concentrations. This blood was immediately transferred in pre-weighed tubes containing 15% sulfosalicylic acid and a known amount of internal standard (~100 μl/ml blood) and mixed well. The internal standard solution contained the following amino acid tracers: L-[U-¹³C₉,¹⁵N]phenylalanine (50 μmol/l) and L-[¹⁵N]threonine (150 μmol/l). After the blood was added, the tubes were weighed again, and the difference was recorded. After centrifugation (3,000 rpm for 20 min at 4°C), the supernatant was frozen for later analysis, which was performed as described previously (30–33). Isotopic enrichment was determined by gas chromatography-mass spectroscopy. Selected ion monitoring was performed for phenylalanine mass-to-charge ratio (*m/z*) 336, 342, and 346 and for threonine *m/z* 404, 405, and 409. The enrichments were expressed as tracer-to-tracee ratios, and appropriate corrections were made for overlapping spectra and the natural distribution of stable isotopes (27, 35).

Blood samples for the determination of leg blood flow were analyzed spectrophotometrically by measuring the ICG dye concentration in serum at a wavelength of 805 nm. Leg plasma flow was calculated from the ICG dye concentrations in the femoral and peripheral veins (20, 21) and converted to blood flow using the hematocrit.

Muscle. About 20–25 mg of the muscle biopsy tissue were weighed, and muscle protein was precipitated with 0.8 ml of 10% perchloroacetic acid. An internal standard solution (2 μl/mg tissue) was added for the determination of free phenylalanine and threonine concentrations in muscle, as described previously (35). In addition, the percent muscle water (H₂O_m) was determined from the weights of wet and dry muscle samples. This percent muscle water was used together with the leg lean mass (LLM) determined from DEXA to calculate a total leg muscle water content, which was taken as the volume of distribution (V_d) for the free phenylalanine in the leg (assuming H₂O density 1.0 g/ml):

$$V_d(\text{liters}) = \text{LLM}(\text{kg}) \times \text{H}_2\text{O}_m(\%)$$

Calculations

Concentrations (nmol/ml) of phenylalanine and threonine in blood were calculated as follows (4, 35):

$$C = Q_{IS}/(V \times E_{IS})$$

where C is the concentration of the amino acid in the blood, Q_{IS} is the amount of internal standard added, V is the volume of the blood, and

E_{IS} is the internal standard tracer-to-tracee ratio in the blood. Because the infused amino acid tracer-to-tracee ratio was also determined by the GC-MS procedure, the contribution of the infused L-[ring- $^{13}C_6$]phenylalanine and L-[U- $^{13}C_4$, ^{15}N]threonine to the blood phenylalanine and threonine concentrations was added to the concentration as determined by the above formula. The concentration of the amino acids in muscle water was also determined by using the above formula.

Arteriovenous balance calculations. Amino acid NB across the leg was calculated as follows:

$$NB = (C_a - C_v) \times BF$$

where C_a and C_v are the amino acid concentrations in the femoral artery and vein, respectively, and BF is the leg blood flow. At each time point, NB defines the sum of uptake (+) and release (-) of an amino acid across the leg. The blood amino acid rates of disappearance from the artery (R_d) and appearance to the vein (R_a) were calculated as follows:

$$R_d = [(E_a \times C_a) - (E_v \times C_v)] \times BF/E_a$$

$$R_a = R_d - NB$$

where E_a and E_v are the blood amino acid enrichments, expressed as tracer-to-tracee ratio, in the femoral artery and vein, respectively. Calculated amino acid kinetics data were expressed relative to the leg volume ($nmol \cdot min^{-1} \cdot 100 ml \text{ leg volume}^{-1}$). Data are presented as average values of the prephenylalanine and postphenylalanine ingestion periods.

The amount (nmol) of free phenylalanine residing in the leg muscle (relative to basal) during the postphenylalanine ingestion period was calculated as follows:

$$(\text{post-Phe}_m - \text{basal-Phe}_m) \times V_d \times 1,000$$

where post-Phe_m (nmol/ml) is the end-of-study muscle free phenylalanine concentration and basal-Phe_m (nmol/ml) is the basal muscle free phenylalanine concentration. Because the duration of the postphenylalanine ingestion period and the leg volume is known, the average rate of phenylalanine movement in the leg after the phenylalanine ingestion can be calculated for a given leg volume ($nmol \cdot min^{-1} \cdot 100 ml \text{ leg volume}^{-1}$). This value is then subtracted from the average phenylalanine NB calculated during the postphenylalanine ingestion period (measured NB_{Phe}) to provide a corrected value for phenylalanine NB during that same period (corrected NB_{Phe}).

A predicted phenylalanine NB (predicted NB_{Phe}) for the postphenylalanine ingestion period based on the measured response of the threonine NB at the same time period (post- NB_{Thr}) and the measured NB for phenylalanine (pre- NB_{Phe}) and threonine (pre- NB_{Thr}) at the baseline was calculated as follows: $\text{measured } NB_{Phe} = (\text{pre-}NB_{Phe} \times \text{post-}NB_{Thr}) / \text{pre-}NB_{Thr}$. The threonine NB was considered to reflect the tissue muscle protein balance, since the steady state in blood and muscle threonine concentrations was not disrupted.

Statistical Analysis

Data were analyzed using ANOVA with repeated measures followed by Dunnett's post hoc tests to determine changes from baseline values. Bland-Altman plots were constructed for phenylalanine NB to assess the degree of agreement of the predicted NB with the measured NB as well as the corrected NB (6). All data are expressed as means \pm SE, and a P value ≤ 0.05 was considered statistically significant.

RESULTS

Blood Flow

The blood flow to the leg was $3.29 \pm 0.44 ml \cdot min^{-1} \cdot 100 ml \text{ leg volume}^{-1}$ before the phenylalanine ingestion and $3.73 \pm$

$0.73 ml \cdot min^{-1} \cdot 100 ml \text{ leg volume}^{-1}$ after phenylalanine ingestion. There were no differences in the mean blood flow rates before and after phenylalanine ingestion ($P > .05$). A single value representing the average of the two blood flow values for these two periods was used for the calculation of muscle amino acid kinetics in each subject to decrease the variability of calculated parameters.

Blood and Muscle Phenylalanine and Threonine Data

Bolus ingestion of 0.5 g phenylalanine caused a twofold increase in arterial blood phenylalanine concentration, which reached a peak at 30 min. Thereafter, phenylalanine concentration decreased steadily but did not return to its baseline value by the time that the study was terminated (Fig. 1B). Femoral venous phenylalanine concentration followed the same pattern as that of the arterial phenylalanine concentration and was consistently higher than the corresponding concentration in the artery, with the exception of 15 and 30 min after the phenylalanine ingestion, when the increase in the arterial concentration was more rapid than that of the venous concentration. The arterial and venous concentrations of the amino acid threonine remained constant throughout the study (Fig. 1A). The variability of individual blood enrichments of phenylalanine and threonine was $<10\%$ of their mean plateau value.

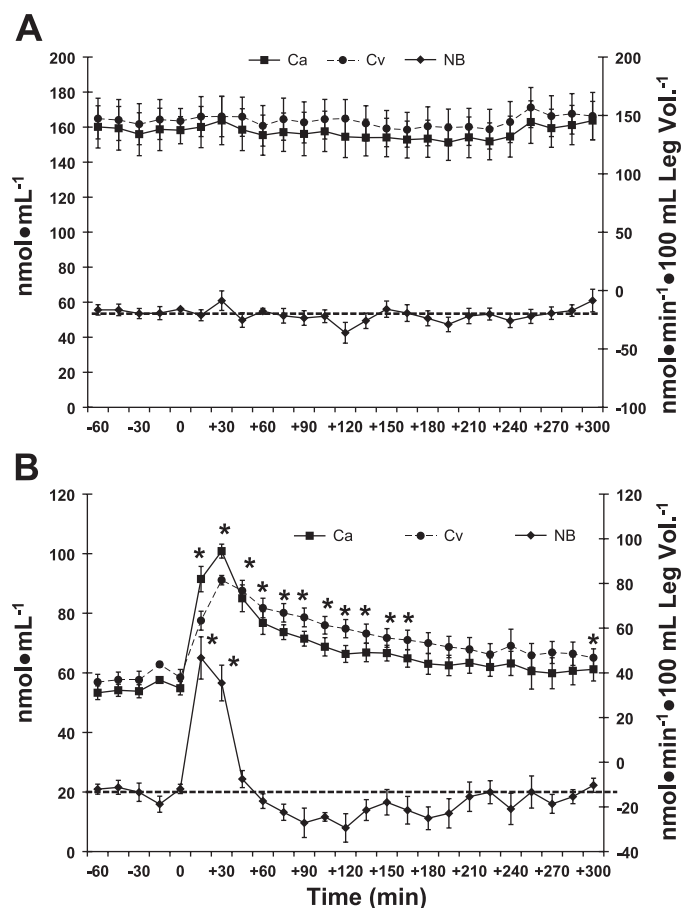


Fig. 1. Threonine (A) and phenylalanine (B) arterial (C_a) and venous (C_v) concentrations in $nmol/ml$ and leg arteriovenous net balance (NB) in $nmol \cdot min^{-1} \cdot 100 ml \text{ leg vol}^{-1}$ after phenylalanine bolus ingestion at 0 min. $*P < 0.05$ compared with baseline (shown only for C_a and NB).

Mean values for the muscle water content (%muscle wt) did not change significantly over the course of the experiment (baseline: 83.1 ± 0.8 ; 1 h: 82.1 ± 1.8 ; end of study: 84.2 ± 1.3 ; $P > 0.05$). Muscle free phenylalanine concentration was increased at 1 h after the phenylalanine ingestion and at the time that the study was terminated compared with baseline ($P < 0.05$; Fig. 2B). There were no differences in the concentrations of muscle free threonine between the time points measured during the study ($P > 0.05$; Fig. 2A).

R_d and R_a of Phenylalanine and Threonine

Phenylalanine R_d increased significantly during the first hour after the phenylalanine ingestion and was numerically lower than the baseline phenylalanine R_d for the remainder of the study (data not shown). The average leg phenylalanine R_d for the entire period after phenylalanine ingestion was not different from baseline ($P > 0.05$; Table 1). On the other hand, the average leg threonine R_d for the entire period after phenylalanine ingestion was lower than baseline ($P < 0.05$; Table 1).

Similar to threonine R_d , the average threonine R_a during the period that followed the phenylalanine ingestion decreased compared with the baseline, but the corresponding value for phenylalanine did not change (Table 1).

Phenylalanine and Threonine NB

During the period before phenylalanine ingestion, there was a net release of both phenylalanine (-13.1 ± 3.0 nmol·min⁻¹·

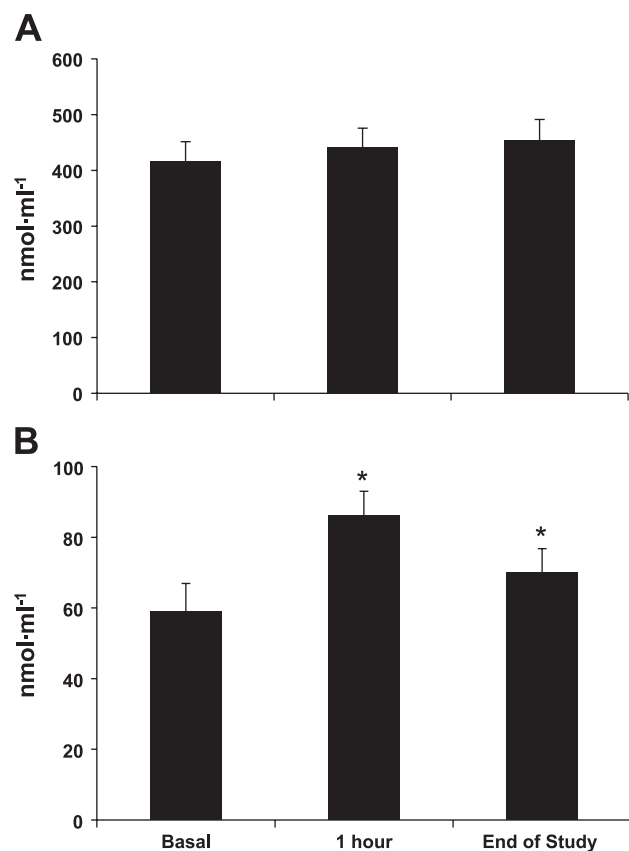


Fig. 2. Muscle free threonine (A) and phenylalanine (B) concentrations in the basal state and after ingestion of phenylalanine. * $P < 0.05$ compared with baseline.

Table 1. Phenylalanine and threonine kinetics across the leg before and after phenylalanine ingestion

	Pre-Phe	Post-Phe
Thr NB	-17.1 ± 3.9	$-22.1 \pm 3.5^*$
Phe NB	-13.1 ± 3.0	-13.5 ± 2.9
Thr R_d	108.7 ± 12.9	$89.6 \pm 12.8^*$
Phe R_d	35.9 ± 4.4	38.0 ± 6.9
Thr R_a	126.1 ± 15.4	$111.7 \pm 15.1^*$
Phe R_a	49.0 ± 7.1	51.4 ± 9.3

Values are means \pm SE. Units are nmol·min⁻¹·100 ml leg vol⁻¹. R_d , rate of disappearance; R_a , rate of appearance; NB, net balance. *Significantly different from Pre-Phe ($P < 0.05$).

100 ml leg volume⁻¹) and threonine (-17.1 ± 3.9 nmol·min⁻¹·100 ml leg volume⁻¹) from the leg. The average response for the entire period after the phenylalanine ingestion showed a net release of both phenylalanine and threonine from the leg. However, the net release of phenylalanine did not change compared with baseline, whereas that of threonine decreased ($P < 0.05$; Table 1). The net release of phenylalanine was significantly higher than the corresponding value predicted based on the threonine NB response before and after phenylalanine ingestion (Fig. 3).

When the phenylalanine NB was corrected for the rate of phenylalanine (2.7 ± 0.7 nmol·min⁻¹·100 ml leg volume⁻¹), contributing to the increase in the concentration of free phenylalanine within its estimated volume of distribution in the leg (7.8 ± 0.5 liters), the net release of phenylalanine after the phenylalanine ingestion decreased compared with baseline ($P < 0.05$). This corrected value for phenylalanine NB was not different from that predicted from the threonine NB (Fig. 3).

Figure 4 shows the Bland-Altman plots, which were used to assess the degree of agreement of measured phenylalanine NB, as well as corrected NB, with that predicted based on the threonine NB. The closer the points are located to the horizontal axis, the better the agreement between the phenylalanine NB values. The mean difference between measured phenylalanine NB and predicted phenylalanine NB was 3.6 ± 0.5 nmol·min⁻¹·100 ml leg volume⁻¹, and this difference was significantly different from zero based on the

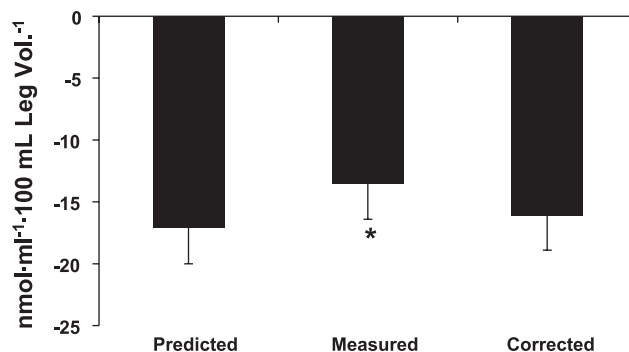


Fig. 3. Leg arteriovenous NB for phenylalanine (nmol·min⁻¹·100 ml leg vol⁻¹), measured after the ingestion of phenylalanine (Measured); predicted based on the threonine and phenylalanine NB values before the phenylalanine ingestion together with the threonine NB after the phenylalanine ingestion (Predicted); and adjusted for the phenylalanine NB associated with the increase in the concentration of free phenylalanine within its volume of distribution in the leg after phenylalanine ingestion (Corrected). * $P < 0.05$ compared with predicted and corrected.

95% limits of agreement shown in Fig. 4A. However, the mean difference in the phenylalanine NB between corrected and predicted values was not significantly different from zero (Fig. 4B).

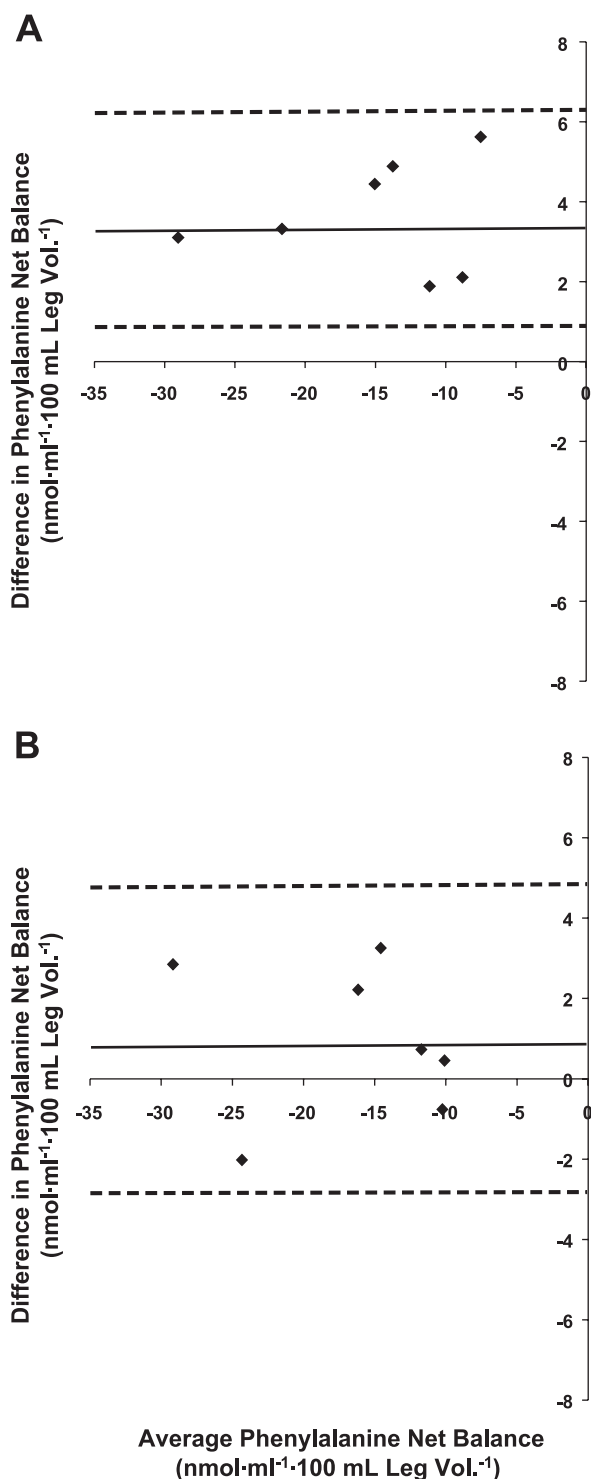


Fig. 4. Bland-Altman plots to assess the agreement of measured (A) and corrected (B) leg arteriovenous phenylalanine NB with the predicted phenylalanine NB after phenylalanine ingestion (measured, corrected, and predicted as defined in Fig. 3). In each plot, the differences between the phenylalanine NB values (e.g., measured NB - predicted NB) are plotted against the averaged phenylalanine NB values [e.g., (measured NB + predicted NB)/2]. Dotted lines represent $1.96 \cdot SD$ about the mean.

DISCUSSION

As expected, our results show that changes in the arteriovenous NB of phenylalanine after a bolus ingestion of phenylalanine do not parallel those of threonine (Fig. 1) and that this dissociation between phenylalanine and threonine NB responses can be attributed to the accumulation of free phenylalanine within its volume of distribution in the leg. The limitation associated with the influence of the volume of distribution of a substance on its reappearance in the vein from the artery in the case of arteriovenous balance measurements has been discussed previously (24). This limitation is documented in the present study in the first arteriovenous blood sample after the bolus ingestion of phenylalanine when the arterial concentration of phenylalanine increased $\sim 67\%$ compared with the $\sim 32\%$ increase in venous concentration (15 min; Fig. 1B). In a physiological circumstance similar to the present study, movement of phenylalanine due to altered transmembrane concentration gradients cannot be presumed to correspond in time to acute changes in muscle protein NB.

In the present study, threonine was used as an independent amino acid tracer to determine the validity of the phenylalanine tracer in determining muscle protein NB. Because phenylalanine (26, 34) and threonine (8, 12, 14) are not metabolized to any substantial extent in the muscle, their arteriovenous NB should provide the same information with regard to the arteriovenous NB of muscle protein. Because phenylalanine and threonine, however, are incorporated into skeletal muscle proteins in different amounts (11), their arteriovenous NB responses are not directly comparable. Despite that, their relative arteriovenous kinetics (e.g., ratio of phenylalanine NB to threonine NB) should remain constant over time irrespective of any changes in the absolute amounts of phenylalanine and threonine retained in muscle proteins. This allows prediction of the arteriovenous phenylalanine NB based on the arteriovenous threonine NB when measured during a steady state in the free threonine concentrations and irrespective of any changes in free phenylalanine concentrations at the same time period.

When the measured response was calculated as an average value for the entire period that followed the phenylalanine bolus ingestion, the phenylalanine NB was statistically different from the NB predicted based on the threonine kinetics (Fig. 3). More specifically, as shown in the Bland-Altman analysis plot in Fig. 4A, the measured phenylalanine NB overestimated the response of muscle protein NB when contrasted to the muscle protein NB predicted from the threonine kinetics. This overestimation in muscle protein NB can be explained by the accumulation of free phenylalanine within the leg muscle (Fig. 2). When the phenylalanine NB responses were corrected for the rate of accumulation of a free phenylalanine within the corresponding volumes of distribution in the leg, the phenylalanine NB responses were not significantly different from those predicted from the threonine NB responses (Figs. 3 and 4B).

The principal goal of the present study was to describe a method that can be used to evaluate muscle protein kinetics during a nonsteady state in the concentrations of free phenylalanine in blood and muscle. However, based on the threonine NB, it can be argued from the findings of the present study that a bolus ingestion of 0.5 g phenylalanine does not have any stimulatory effects on the accretion of muscle proteins in the

leg. An unexpected finding in the present study is the alteration of transmembrane rates of transport in leg threonine after the bolus ingestion of phenylalanine. The overall leg threonine R_a decreased (~11%) consistently in all subjects after the phenylalanine ingestion. Although the exact mechanisms cannot be explained based on the present study design, this decrease may be a compensatory response to prevent a decrease in muscle intracellular free threonine after a comparably larger decrease in the leg threonine R_d (~18%), since the concentrations of free amino acids in the muscle cells are very tightly regulated (23). In part, the decrease in leg threonine R_d could be related to the increase in blood phenylalanine concentration and the competition between the two amino acids for muscle cell transporters, since both amino acids share to some extent the same transport mechanisms (10). Finally, the overall decrease in threonine NB could represent a decline in muscle protein synthesis due to the lack of activity during the study.

When the correction for the phenylalanine NB described in this paper was used, the phenylalanine NB response indicated muscle protein NB similar to that indicated from the threonine NB. The correction was based on the determination of leg lean mass by DEXA, which provides a valid and reliable estimation of limb muscle mass (16), together with the assumption that leg protein balance is mainly a reflection of muscle protein balance. The later assumption is reasonable under normal physiological conditions (5). In the absence of an accurate estimation of leg lean mass and/or the circumstance of an abnormal (e.g., clinical) situation, assumptions regarding these factors could amplify variability. In that case, it may be more appropriate, if possible, to carry out the experiment for sufficient time for the basal muscle free phenylalanine concentration to be reestablished to make valid conclusions regarding muscle protein kinetics. Although the latter could also be appropriate to reduce the variability introduced by the measurement of muscle tissue water content, waiting for basal muscle free phenylalanine concentration to be reestablished may not be practical (e.g., time required may not be known) or in some instances may be undesirable when short-lived effects on muscle protein metabolism are evaluated.

There have been previous efforts to correct the arteriovenous phenylalanine NB response in the circumstance in which there is an increase in muscle free phenylalanine concentration after the ingestion of drinks containing phenylalanine (25, 31). In those circumstances, the leg volume of distribution for phenylalanine was taken to represent a fixed percent of leg volume (e.g., 60%). In the present study, correlation analysis between the leg volume of distribution (7.8 ± 0.5 liters), as estimated from leg lean mass, and a corresponding value calculated based on 60% of the leg's volume (7.2 ± 0.2 liters) yields $R^2 = 0.03$ ($P > 0.05$). Although for practical purposes the muscle in the leg can be assumed to represent a given volume of the total leg (13), use of such estimates results in increased variability with respect to the calculation of volume of distribution estimates for free amino acids in the leg, because they do not take into account individual differences in leg fat mass. Finally, it should be noted that the methodology we have described requires muscle biopsies to determine muscle free amino acid concentrations. When these values cannot be determined, the methodology is not applicable.

In conclusion, we describe for the first time an arteriovenous balance method for the determination of leg muscle protein

balance during non-steady-state muscle amino acid concentrations, a method that is based on the determinations of leg lean mass and the concentration of free amino acids in muscle. This method can be applied when various short-lived nutritional effects on skeletal muscle are studied and when physiological perturbations in the concentration of blood and muscle amino acids are expected, as in the case immediately after meal ingestion.

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