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Measurement of protein fractional synthesis and breakdown rates in the skin of rabbits using a subflooding dose method

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Abstract

The flooding dose method continues to be useful in measuring protein fractional synthetic rate (FSR) in a tissue. However, flooding of free amino acid pools eliminates enrichment difference between plasma and tissue free amino acid pools, which makes it impossible to concomitantly measure protein fractional breakdown rate (FBR). We hypothesized that a subflooding dose of an amino acid reduces the enrichment difference between plasma and tissue free amino acid pool to a minimal measurable level, thus allowing concomitant measurement of protein FSR and FBR. Phenylalanine (40% enriched) at 50 mg/kg was intravenously injected as a bolus in 6 anesthetized rabbits. Arterial blood and chest skin samples were taken before the injection and for 120 minutes after the injection. Fractional breakdown rate of skin protein calculated from 15-60-120–minute sampling times was 11.3%/d ± 2.0%/d, which was close (P = .66) to the corresponding FSR of 10.0%/d ± 2.3%/d. The subflooding dose injection did not disturb the FBR approach because modifications on the FBR equation to account for the changes of plasma phenylalanine concentration resulted in the same value (11.5%/d ± 1.4%/d). The FBR was positively correlated with the FSR (r = 0.80, P < .05). These findings indicate maintenance of protein mass in the skin, which is a metabolic characteristic of the skin. This subflooding dose method provides a methodological choice to concomitantly measure both FSR and FBR in a tissue.

1. Introduction

The flooding dose method has been used for several decades to determine the fractional synthesis rate (FSR) of tissue protein [1-5]. This method involves injection of a large bolus dose of an amino acid along with an isotopically labeled counterpart to achieve an equilibration of isotopic enrichment (or specific radioactivity) in the tissue aminoacyl–transfer RNA (tRNA), tissue free amino acid, and plasma pools. Thus, the plasma or tissue free enrichment (or specific radioactivity) can be used to represent aminoacyl-tRNA enrichment in the calculation of protein FSR. This method continues to be useful in measuring tissue protein synthesis, especially when the true precursor pool is not accessible [6-8], the tissue free amino acid pool is not a good surrogate of the true precursor [9], or a continuous tracer infusion is difficult or impractical [10-12].

We have previously published isotopic methods for measurement of muscle protein fractional breakdown rate (FBR) by means of measuring enrichment decay either after stopping a constant tracer infusion or after a pulse tracer injection [13,14]. The FBR measurement is a reverse application of the precursor-product principle as conventionally applied to protein kinetics because it regards tissue free amino acids as product and both tissue protein breakdown and arterial amino acid transport inflow as precursors. The calculation requires an enrichment gradient from plasma to the intracellular pool to calculate tracee contribution from these 2 precursors. Therefore, theoretically, if there is measurable difference of enrichment (or specific radioactivity) between these precursor pools, the FBR measurement is applicable.

The FBR measurement can be combined with the FSR measurement from either constant tracer infusion or pulse tracer injection to calculate both the FBR and FSR [13,14]. The pulse tracer injection involves pure tracer injection at a dose much less than the flooding dose [14]. After a flooding dose, the plasma, tissue free amino acid, and aminoacyl-tRNA pools are equilibrated, which makes it impossible to measure protein FBR. If a dose of tracer and tracee mixture is large enough to sufficiently reduce the enrichment (or

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specific radioactivity) gradient but not sufficient to equilibrate the plasma and tissue free amino acid pools, the FBR measurement would be possible. Thus, we propose this approach as a subflooding dose method. The goal of this method is to preserve the advantages of the flooding dose method for protein FSR measurement while simultaneously enabling measurement of protein FBR.

The present experiment was designed to measure protein FSR and FBR in the skin of rabbits using a subflooding dose of stable isotope-labeled phenylalanine (Phe) along with unlabeled Phe. Previous publications demonstrated that muscle free amino acid enrichment is a good surrogate of true precursor (ie, aminoacyl-tRNA) enrichment for calculating muscle protein FSR [15,16]. Thus, our pulse tracer injection method is sufficient in measuring muscle protein FSR and FBR [14]. However, it has been reported that skin free amino acid enrichment may be greater than the corresponding aminoacyl-tRNA enrichment [9]. Thus, we used the skin as the target tissue to test this subflooding dose method.

2. Methods

2.1. Rationale and equation for calculation of skin protein FBR

We previously developed an isotopic method to determine muscle protein FBR based on the decay of enrichment after a constant tracer infusion is stopped [13]. This original method requires infusion of an amino acid tracer (eg, Phe) and measurement of arterial amino acid enrichment and intracellular free amino acid enrichment during isotopic steady state and during the decay period after stopping the tracer infusion. The equation used to calculate FBR is as follows:

$$\mathrm{FBR} = \frac{E_{\mathrm{M}}(t_2) - E_{\mathrm{M}}(t_1)}{P\int_{t_1}^{t_2} E_{\mathrm{A}}(t)dt - (1+P)\int_{t_1}^{t_2} E_{\mathrm{M}}(t)dt}(Q_{\mathrm{M}}/T), \tag{1}$$

where $E_{\mathrm{M}}(t)$ is the intracellular free enrichment at time t, $E_{\mathrm{A}}(t)$ is the arterial enrichment at time t, Q_{M}/T is the ratio of intracellular free content to protein-bound content in muscle, and $P = E_{\mathrm{M}}/(E_{\mathrm{A}} - E_{\mathrm{M}})$, where E_{M} and E_{A} are the intracellular and arterial enrichments at isotopic steady state.

After a subflooding dose injection, an isotopic steady state is not obtained; so it is not possible to calculate the value of P. We shall show a simple way of eliminating P from the above equation and thus removing the requirement of obtaining steady-state measurements. The above equation holds for any 2 time points; so if we choose time points from t_2 and t_3 rather than t_1 and t_2, then the equation becomes

$$\mathrm{FBR} = \frac{E_{\mathrm{M}}(t_3) - E_{\mathrm{M}}(t_2)}{P\int_{t_2}^{t_3} E_{\mathrm{A}}(t)dt - (1+P)\int_{t_2}^{t_3} E_{\mathrm{M}}(t)dt}(Q_{\mathrm{M}}/T). \tag{2}$$

Thus, we have 2 equations and 2 unknowns, that is, FBR and P. If we solve Eq. 2 for P and substitute it into Eq. 1, we obtain the equation

$$\mathrm{FBR} = \frac{[E_{\mathrm{M}}(t_2) - E_{\mathrm{M}}(t_1)]\cdot\int_{t_2}^{t_3}[E_{\mathrm{A}}(t) - E_{\mathrm{M}}(t)]dt - [E_{\mathrm{M}}(t_3) - E_{\mathrm{M}}(t_2)]\cdot\left[\int_{t_1}^{t_2} E_{\mathrm{A}}(t) - E_{\mathrm{M}}(t)dt\right]}{\int_{t_2}^{t_3} E_{\mathrm{M}}(t)dt\cdot\int_{t_1}^{t_2} E_{\mathrm{A}}(t)dt - \int_{t_1}^{t_2} E_{\mathrm{M}}(t)dt\cdot\int_{t_2}^{t_3} E_{\mathrm{A}}(t)dt}(Q_{\mathrm{M}}/T). \tag{3}$$

Therefore, a measurement at isotopic steady state is not required if the arterial and intracellular enrichments are measured at 3 time points. This is the same rationale and equation we use for the pulse tracer injection [14], except that with the subflooding dose method both tracer and tracee are injected so that the ratio of Q_{m}/T in Eq. 3 is a varying value. Therefore, the mean Q_{m}/T from the 3 time points is used for calculation.

The varying Q_{m}/T value represents a physiologic nonsteady state that violates the assumptions of the traditional FBR approach. Thus, it is necessary to assess if the use of mean Q_{m}/T from the 3 time points is acceptable. To this end, we have modified the FBR formula to account for the nonsteady state. The modified FBR formula is derived with the addition of 2 new assumptions to the traditional FBR approach, namely, that inward transport of amino acids is proportional to the arterial amino acid concentration and that protein breakdown is constant over the period when the measurement is made. If these assumptions hold, the FBR can be calculated by the following formula (see Appendix for derivation):

$$\mathrm{FBR} = \frac{[E_{\mathrm{M}}(t2) - E_{\mathrm{M}}(t1)]\cdot\int_{t2}^{t3}[E_{\mathrm{A}}(t) - E_{\mathrm{M}}(t)]\cdot C_{\mathrm{A}}(t)R(t)dt - [E_{\mathrm{M}}(t3) - E_{\mathrm{M}}(t2)]\cdot\int_{t1}^{t2}[E_{\mathrm{A}}(t) - E_{\mathrm{M}}(t)]\cdot C_{\mathrm{A}}(t)R(t)dt}{\left(\int_{t2}^{t3} E_{\mathrm{M}}(t)R(t)dt\right)\cdot\left(\int_{t1}^{t2}[E_{\mathrm{A}}(t) - E_{\mathrm{M}}(t)]\cdot C_{\mathrm{A}}(t)R(t)dt\right) - \left(\int_{t1}^{t2} E_{\mathrm{M}}(t)R(t)dt\right)\cdot\left(\int_{t2}^{t3}[E_{\mathrm{A}}(t) - E_{\mathrm{M}}(t)]\cdot C_{\mathrm{A}}(t)R(t)dt\right)}, \tag{4}$$

where $E_M(t)$ is the enrichment of intracellular free amino acids, $E_A(t)$ is the enrichment of arterial amino acids, $C_A(t)$ is the concentration of arterial amino acids, and $R(t)$ is the ratio of bound to free intracellular amino acids at time t.

The FSR of skin protein was calculated by the tracer incorporation method, which is based on the precursor-product principle [17]. The equation is

$$\mathrm{FSR} = \frac{E_{t_2} - E_{t_1}}{E_p(t_2 - t_2) \times (t_2 - t_1)}, \tag{5}$$

where $(E_{t2} - E_{t1})$ is the increment of skin protein enrichment from t_1 to t_2; $E_P(t_2 - t_1)$ is the precursor enrichment calculated from area under the curve of skin free Phe enrichment from t_1 to t_2.

2.2. Experimental procedures

2.2.1. Animals

We used male New Zealand white rabbits (Myrtle's Rabbitry, Thompson Station, TN) weighing about 4.5 kg. The rabbits were housed in individual cages and fed Lab Rabbit chow 5326 (Purina Mills, St Louis, MO) for weight maintenance. This study was approved by the Animal Care and Use Committee of The University of Texas Medical Branch at Galveston.

2.2.2. Isotopes

L-[^{15}N]Phe (99% enriched) was purchased from ISOTEC (Miamisburg, OH), and L-[ring-$^{13}C_6$]Phe (99% enriched) was purchased from Cambridge Isotope laboratories (Woburn, MA).

2.2.3. Experimental design

The anesthetic and surgical procedures were described in our previous publications [18]. In brief, after an overnight food deprivation with free access to water, the rabbits were anesthetized with ketamine and xylazine. The right femoral artery and vein were catheterized via a groin incision. The arterial line was used for blood collection and monitoring of mean arterial blood pressure and heart rate, and the venous line was used for infusion. A tracheal tube was placed via tracheotomy. The hair of skin on the chest was removed with a clipper.

The subflooding dose of Phe was composed of L-[^{15}N]Phe and unlabeled Phe at an enrichment of 0.4 (tracer-tracee ratio). The dose was 50 mg/kg in 15 mL 0.45% sodium chloride and was injected intravenously within 1 minute. In the first 2 rabbits, frequent samples were taken. Arterial blood was drawn before the bolus injection and at 5, 10, 15, 30, 45, 60, 75, 90, 105, and 120 minutes after the injection; skin samples were taken before the injection and at 5, 10, 15, 60, 75, and 120 minutes after the injection. Based on the results from the first 2 rabbits, the sample numbers were reduced to 5 in the later 4 rabbits: before the tracer injection and at 15, 60, 90, and 120 minutes after the injection. Plasma was separated from blood by centrifugation and was stored at −20°C for later analysis. The skin samples were taken with 6-mm biopsy punches, quickly washed in ice-cold saline to remove blood contamination, and immediately frozen in liquid nitrogen. The skin samples were stored at −80°C before later analysis.

Arterial blood pressure, heart rate, chest skin surface, and rectal temperature were maintained stable by adjusting the infusion rates of anesthetics and 0.9% sodium chloride, and a heating lamp. The vital signs were recorded every 15 minutes for 2 hours after Phe injection.

2.2.4. Sample analysis

To 0.25 mL plasma, L-[ring-$^{13}C_6$]Phe (30 nmol/mL) was added for calculation of Phe concentration; the volume of the internal standard solution added to each plasma (0.25 mL) was 0.4 to 0.07 mL to account for the decline of unlabeled Phe concentration after subflooding dose injection. The *t*-butyldimethylsilyl derivatives of amino acids were prepared from the supernatant of plasma [19].

Skin samples were processed as we previously described [20]. In brief, the panniculus carnosus muscle was removed with a surgical blade. L-[Ring-$^{13}C_6$]Phe at 6 nmol/L was added to the skin sample at a ratio of 1 to 1.5 μL to 1 mg wet tissue. The samples were homogenized in 100 g/L perchloric acid. The supernatant and protein precipitate were processed for *t*-butyldimethylsilyl derivatives of amino acids. Another set of protein precipitate was processed for Phe content using L-[ring-$^{13}C_6$]Phe as the internal standard [13,14].

Data were expressed as means ± SE. Isotopic enrichments were expressed as tracer-tracee ratio for FBR calculation and as mole percentage excess for FSR calculation, as required by the methods [13,17]. Differences between 2 groups were evaluated with *t* test. The relation between FSR and FBR was tested with a linear fit. A P value less than .05 was considered statistically significant.

3. Results

The body weight of rabbits was 4.6 ± 0.1 kg. During the 2 hours after the subflooding dose injection, body temperature was 39.2°C ± 0.2°C, chest skin surface temperature was 37.3°C ± 0.2°C, heart rate was 177 ± 9 beats per minute, and mean arterial pressure was 70 ± 2 mm Hg. The rabbits tolerated the subflooding dose of Phe injection well, as the vital signs did not show major changes before and after the injection.

In the first 2 rabbits, we took frequent blood and skin samples after the injection. Plasma Phe enrichment and concentration reached peaks at 5 minutes; thereafter, they declined over time (Fig. 1A and B). The peak plasma Phe concentration was on average 994 nmol/mL. Peak skin free Phe concentration at 10 minutes was 630 nmol/mL. However, skin free Phe enrichment peaked at 15 minutes, then declined over time (Fig. 1A and B). Thus, in the following 4 rabbits, sampling times were reduced: before the injection and at 15, 60, 90, and 120 minutes after the injection. Skin free Phe concentration declined much slower than plasma Phe concentration (Fig. 2A and B). Protein accounted for 32.1% ± 0.6% of wet chest skin and contained 134.3 ± 1.2 μmol of Phe (n = 6), so 1 g of wet skin contained 43.2 μmol Phe. The contents of unlabeled Phe in the free and protein-bound pools were used for the FBR calculation.

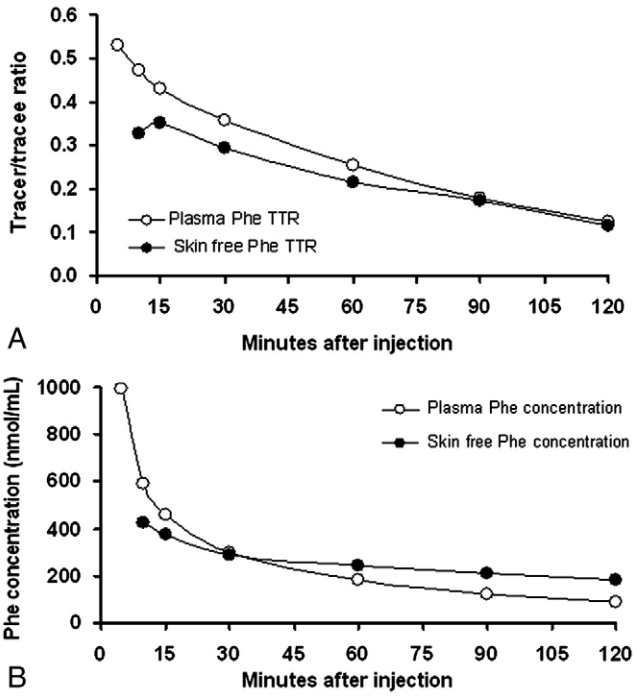

Fig. 1. Phenylalanine enrichment and concentration in plasma and skin free amino acid pools in the first 2 rabbits. A, Whereas Phe enrichment in plasma peaked at 5 minutes, Phe enrichment in the skin free amino acid pool peaked at 15 minutes. B, Phenylalanine concentration in plasma was 994 nmol/mL at 5 minutes, and Phe concentration in the skin free amino acid pool was 428 nmol/mL at 10 minutes. The results from these 2 rabbits were used to adjust sampling times in the next 4 rabbits.

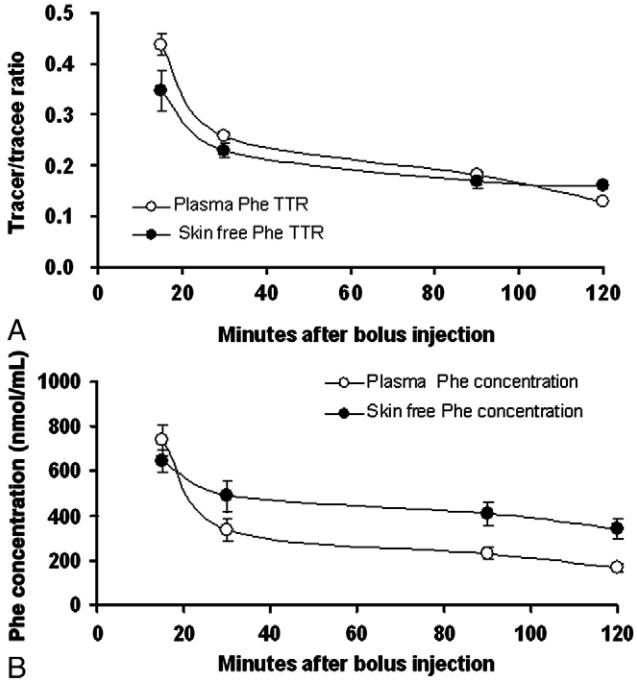

Fig. 2. Phenylalanine enrichment and concentration in plasma and skin free amino acid pools in all 6 rabbits. A, Decay curves of Phe enrichments in plasma and skin free amino acid pools. The highest enrichments were observed at 15 minutes. B, Decay curves of Phe concentrations in plasma and skin free amino acid pools. The greatest concentrations were observed at 15 minutes. The enrichment difference between plasma and skin free amino acid pools was small but measurable. Therefore, both the FBR and FSR were measured from the subflooding dose injection.

The FBR calculation requires 3 time points of sampling. Thus, we calculated FBR from 15-60-90, 15-60-120, and 15-90-120 minutes after the subflooding dose injection (Table 1). Although the mean FSR values from the 3 different combinations of time points were close, the FBR values calculated from 15-60-90 and 15-90-120 minutes varied in some individuals (ie, in rabbits 1, 3, and 6). In contrast, the FBR values calculated from 15-60-120 minutes were consistent and were comparable with the mean values of 3 combinations of sampling times. Thus, we used the FBR

Table 1
Protein FBR in chest skin

	15-60-90 min	15-60-120 min	15-90-120 min	Mean
Rabbit 1	16.5	5.4	2.9	8.3
Rabbit 2	12.0	10.7	8.5	10.4
Rabbit 3	23.9	16.8	16.1	18.9
Rabbit 4	7.6	7.2	7.3	7.4
Rabbit 5	8.2	10.5	11.6	10.1
Rabbit 6	11.0	17.3	20.4	16.2
Mean ± SE	13.2 ± 2.5	11.3 ± 2.0	11.1 ± 2.6	11.9 ± 1.9

Values are means ± SE in percentage per day.

Table 2
Protein FSR in chest skin

	Phe enrichment		Hours of decay	FSR (%/d)
	Free amino acid pool	Protein-bound pool		
Rabbit 1	19.43%	0.0944%	1.75	6.7
Rabbit 2	16.21%	0.1461%	1.75	12.4
Rabbit 3	14.52%	0.1473%	1.75	13.9
Rabbit 4	17.37%	0.0718%	1.75	5.7
Rabbit 5	22.00%	0.0516%	1.75	3.2
Rabbit 6	18.20%	0.2574%	1.75	18.0
Mean ± SE	18.20% ± 1.10%	0.1281% ± 0.0303%	1.75	10.0 ± 2.3

Phenylalanine enrichments are expressed as mole percentage excess.

values calculated from 15-60-120 minutes (11.3%/d ± 2.0%/d) to represent protein FBR in the skin. The FBR values calculated from Eq. 4, which was modified to account for the physiologic nonsteady state, were 11.5%/d ± 1.4%/d, identical to the values calculated from Eq. 3.

Protein FSR was calculated from 15 to 120 minutes after the bolus injection, using the area under curve of free Phe enrichment at 15-, 60-, 90-, and 120-minute time points (Table 2). Protein FSR in the chest skin was 10.0%/d ± 2.3%/d. There was no significant difference (P = .66) between protein FSR and FBR in the skin. Furthermore, there was a positive correlation between FSR and FBR calculated either from the original equation (Eq. 3) (r = 0.80, P < .05; Fig. 3A) or from the modified equation (Eq. 4) (r = 0.74, P < .05; Fig. 3B).

4. Discussion

Using the subflooding dose method, skin protein FSR and FBR were 10.0%/d ± 2.3%/d and 11.3%/d ± 2.0%/d, respectively (P = .66). The comparable skin protein FSR and FBR indicate that the protein mass in the skin was maintained in the fasting state. This finding is consistent with the observation that skin generally maintains its protein mass under a variety of hormonal and nutritional conditions, a conclusion drawn from our previous experiment in which an arteriovenous balance method was used [21]. The positive correlation between skin protein FSR and FBR further supports the maintenance of skin protein mass. Because the FBR calculation is based on measuring true precursor and product without a surrogate, it measures the true breakdown rate of tissue protein. The comparable FSR and FBR values in a circumstance in which we would expect the values to be comparable suggest the same reliability of the FSR measurement. Thus, the subflooding dose method successfully measured protein FSR and FBR in the skin.

Measurement of tissue protein FSR using the flooding dose method requires that intracellular free amino acid enrichment rises rapidly after injection and remains elevated and stable over the course of the protein synthesis measurement period [22]. To achieve this goal, a large dose of amino acid is required. For example, Davis et al [23] injected a flooding dose of Phe in neonatal pigs that provided 1.5 mmol Phe per kilogram of body weight (ie, 247.5 mg/kg). Thirty minutes after the flooding dose injection, muscle phenylalanyl-tRNA specific radioactivity was 98% that in the blood, indicating satisfactory equilibration of tracer in these pools. Such equilibration eliminates the possibility of concomitant measurement of protein FBR because the enrichment difference between blood and tissue free amino acid pools is not measurable. In the present study, the subflooding dose of 50 mg/kg Phe markedly reduced the enrichment difference from plasma to skin free amino acid pool (Fig. 1). At 30 minutes after the injection, skin free Phe enrichments were 89% of the plasma enrichment, smaller than 98% with a flooding dose [23] but greater than 78% with constant tracer infusion [20]. This suggests a subflooding of the precursor pools,

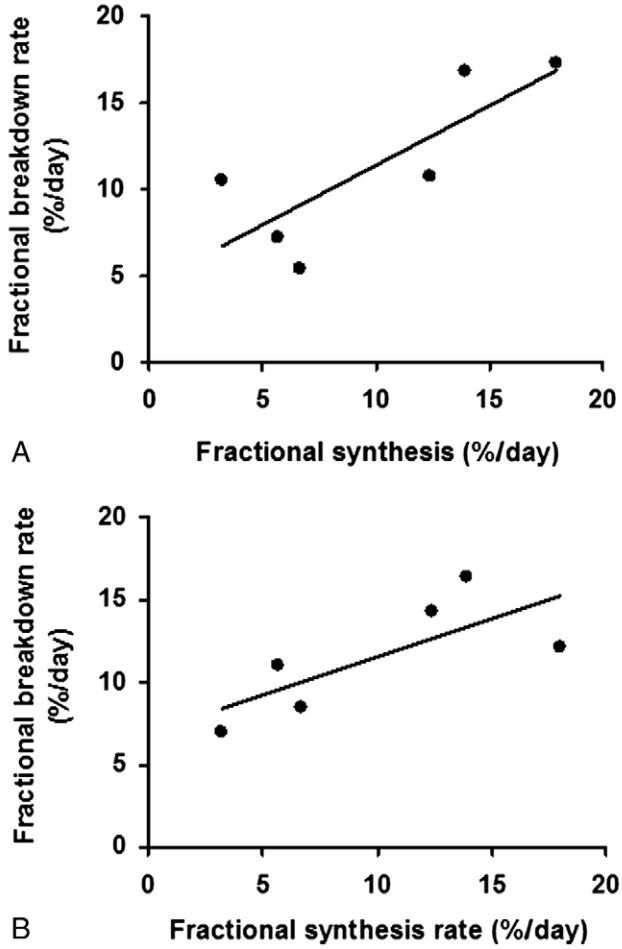

Fig. 3. There was a positive correlation between skin protein FSR and FBR calculated from either (A) the original equation (Eq. 3) (r = 0.80, P < .05) or (B) the modified equation (Eq. 4) (r = 0.74, P < .05). The correlation and comparable FSR and FBR values support the notion that both the original and modified FBR calculations were valid.

which reduces the enrichment difference to a minimal measurable level.

After the subflooding dose injection, the peak plasma Phe concentration at 5 minutes was 994 nmol/mL, 13.7-fold that of 72.5 nmol/mL in the postabsorptive state [14]. Such an increase in plasma Phe concentration might have flooded the free amino acid pool in muscle. In fact, in some publications, the flooding dose used for measurement of muscle protein FSR was 50 mg/kg of leucine [24,25]. This dose increased plasma leucine concentration approximately 6-fold [24]. That flooding dose effectively minimized the specific radioactivity (or enrichment) between blood and muscle free amino acid pools. Considering the smaller pool size of Phe than leucine and higher plasma Phe concentration than that after reported leucine flooding, the subflooding dose of Phe injection might have flooded the muscle free amino acid pool. Thus, the tissue specificity needs to be considered when the subflooding dose method is used.

After the subflooding dose injection, the changing concentration of plasma unlabeled Phe violated the assumption of physiologic steady state in our original FBR approach [9]. To assess the validation of the FBR approach, we have modified the FBR approach to account for the physiologic nonsteady state. The identical FBR values obtained from the original and modified equations support the acceptability of the original FBR approach after the subflooding dose injection provided the mean intracellular free to protein-bound tracee ratio from the 3 sampling time points is used for calculation. We previously reported that protein FSR and FBR were 0.169%/h ± 0.055%/h and 0.260%/h ± 0.011%/h (means ± SEM, n = 5) from rat back skin using the constant tracer infusion [26] and were 0.543%/h ± 0.218%/h and 0.507%/h ± 0.157%/h (means ± SEM, n = 5) from dog leg skin using the arteriovenous balance method [27]. The results from the subflooding dose method are comparable with those previous data with respect to the magnitude of skin protein turnover and coefficient of variation.

In summary, a bolus injection of an amino acid tracer along with its tracee to subflood the free amino acid pools enables measurements of both the protein FSR and FBR in a tissue. The goal of this approach is to decrease the difference in enrichment (or specific radioactivity) between blood and tissue free amino acid pools. Whereas this approach has conventionally used a large dose of tracee and tracer (ie, "flooding dose"), in this article, we have described the use of a "subflooding" dose of tracer and tracee. The subflooding dose minimizes the enrichment differences between blood and intracellular amino acids, thereby minimizing uncertainty regarding precursor enrichment for calculation of FSR while simultaneously enabling the measurement of tissue FBR.

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Appendix A. Derivation of FBR formula in the nonsteady state:

The following is the formula for calculating the FBR of tissue protein:

$$\mathrm{FBR}=\frac{[E_M(t_2)-E_M(t_1)]\cdot\int_{t_2}^{t_3}[E_A(t)-E_M(t)]\cdot C_A(t)R(t)dt-[E_M(t_3)-E_M(t_2)]\cdot\int_{t_1}^{t_2}[E_A(t)-E_M(t)]\cdot C_A(t)R(t)dt}{\left(\int_{t_2}^{t_3}E_M(t)R(t)dt\right)\cdot\left(\int_{t_1}^{t_2}[E_A(t)-E_M(t)]\cdot C_A(t)R(t)dt\right)-\left(\int_{t_1}^{t_2}E_M(t)R(t)dt\right)\cdot\left(\int_{t_2}^{t_3}[E_A(t)-E_M(t)]\cdot C_A(t)R(t)dt\right)},\tag{A1}$$

where $E_M(t)$ is the enrichment of intracellular free amino acids, $E_A(t)$ is the enrichment of arterial amino acids, $C_A(t)$ is the concentration of arterial amino acids, and $R(t)$ is the ratio of bound to free intracellular amino acids at time t. The purpose of this Appendix is to derive Eq. A1.

We will assume that the intracellular free amino acid pool acts like a single pool and that the intracellular free amino acid pool size (denoted as $Q_M[t]$) is changing at rate $Q_M'(t)$. We will assume that the rate that the intracellular free amino acid pool size is changing is equal to the rate that unlabeled amino acids enter the intracellular free pool via protein breakdown (denoted as PB) and inward transport (denoted as $F_{M,A}$) minus the rate that unlabeled amino acids exit the intracellular free pool via protein synthesis (denoted as PS) and outward transport (denoted as $F_{V,M}$), as expressed by the following equation:

$$Q_M'(t)=PB(t)+F_{M,A}(t)-PS(t)-F_{V,M}(t)\tag{A2}$$

We assume here that there is no other source of amino acids. We will define the variable $p(t)$ by the formula

$$p(t)=F_{M,A}(t)/PB(t).\tag{A3}$$

Eq. A3 can be rearranged to give

$$F_{M,A}(t) = p(t)PB(t), \tag{A4}$$

so Eq. A2 can be written as

$$Q_M'(t) = PB(t) + p(t)PB(t) - PS(t) - F_{V,A}(t) \tag{A5}$$

or

$$PS(t) + F_{V,M}(t) = (1 + p(t))PB(t) - Q_M'(t). \tag{A6}$$

We will assume that tracer only enters the intracellular space from blood. Under most circumstances, the enrichment of bound protein will be a thousand-fold lower than the blood enrichment; so this is a reasonable assumption. The rate that tracer enters into the intracellular space at a given time t will thus be equal to the rate that tracee is transported into the cell times the tracer to tracee ratio of blood amino acids, that is, $E_A(t) \cdot F_{M,A}(t)$. Likewise, the rate that tracer leaves the intracellular space (via both transport and incorporation into protein) at a given time t will be equal to the rate that tracee leaves the intracellular space times the tracer to tracee ratio of intracellular amino acids, that is, $E_M(t) \cdot [PS(t) + F_{V,M}(t)]$.

The rate that the intracellular tracer pool size is changing (denoted as $q_M'[t]$) will be equal to the rate that tracer enters the intracellular pool minus the rate that tracer leaves the intracellular pool; that is,

$$q_M'(t) = E_A(t)F_{M,A} - E_M(t)\left(PS(t) + F_{V,M}(t)\right). \tag{A7}$$

Now, because the intracellular enrichment is by definition equal to the ratio of the tracer to tracee intracellular pool sizes (ie, $E_M[t] = q_M[t]/Q_M$), we can conclude that

$$q_M(t) = E_M(t)Q_M(t). \tag{A8}$$

Differentiating both sides of Eq. A8 with respect to time gives

$$q_M'(t) = E_M'(t)Q_M(t) + E_M(t)Q_M'(t). \tag{A9}$$

So combining Eqs. A7 and A9 gives

$$E_M'(t)Q_M + E_M(t)Q_M'(t) = E_A(t)F_{M,A}(t) - E_M(t)\left(PS(t) + F_{V,M}(t)\right). \tag{A10}$$

If we substitute Eqs. A4 and A6 into Eq. A10, then

$$E_M'(t)Q_M(t) + E_M(t)Q_M'(t) = E_A(t)p(t)PB(t) - E_M(t)[(1 + p(t))PB(t) - Q_M'(t)]. \tag{A11}$$

If we subtract $E_M(t) \cdot Q_M'(t)$ from both sides of the above equation and then divide both sides of Eq. A11 by $Q_M(t)$, we get

$$E_M'(t) = E_A(t)p(t)PB(t)/Q_M(t) - E_M(t)(1 + p(t))PB(t)/Q_M(t). \tag{A12}$$

To proceed further, we will assume that protein breakdown is constant and that the inward transport is proportional to the arterial concentration (denoted as $C_A[t]$). Because by definition $p(t) = F_{M,A}(t)/PB(t)$, $p(t)$ must be proportional to the arterial concentration; so we will define the proportionality constant (denoted as b) to satisfy the relationship

$$p(t) = bC_A(t). \tag{A13}$$

Therefore, Eq. A12 can be written as

$$E_M'(t) = E_A(t)bC_A(t)PB/Q_M(t) - E_M(t)[1 + bCA(t)]PB/Q_M(t). \tag{A14}$$

If we integrate both sides of Eq. A14 from time t_1 to time t_2, we get

$$E_M(t_2) - E_M(t_1) = b \cdot PB \cdot \int_{t_1}^{t2} E_A(t) \cdot C_A(t)/Q_M(t)dt - PB \cdot \int_{t_1}^{t2} [1 + b \cdot C_A(t)] \cdot E_M(t)/Q_M(t)dt, \tag{A15}$$

which can be rearranged to get

$$E_M(t_2) - E_M(t_1) = PB \cdot \left(b \cdot \int_{t_1}^{t_2} [E_A(t) - E_M(t)] \cdot C_A(t)/Q_M(t)dt - \int_{t_1}^{t_2} E_M(t)/Q_M(t)dt \right), \tag{A16}$$

The above equation applies for any 2 time points, so it also applies for time points t_2 and t_3:

$$E_M(t_3) - E_M(t_2) = PB \cdot \left(b \cdot \int_{t_2}^{t_3} [E_A(t) - E_M(t)] \cdot C_A(t)/Q_M(t)dt - \int_{t_2}^{t_3} E_M(t)/Q_M(t)dt \right). \tag{A17}$$

If we solve Eq. A17 for b, we obtain

$$b = \left(E_M(t_3) - E_M(t_2) + PB \int_{t_2}^{t_3} E_M(t)/Q_M(t)dt \right) / \left(PB \cdot \int_{t_2}^{t_3} [E_A(t) - E_M(t)] \cdot C_A(t)/Q_M(t)dt \right). \tag{A18}$$

If we substitute Eq. A18 into Eq. A16, we get

$$\begin{aligned} E_M(t_2) - E_M(t_1) = & -PB \\ & \cdot \int_{t_1}^{t_2} E_M(t)/Q_M(t)dt + \left(E_M(t_3) - E_M(t_2) + PB \int_{t_2}^{t_3} E_M(t)/Q_M(t)dt \right) / \left(\int_{t_2}^{t_3} [E_A(t) - E_M(t)] \cdot C_A(t)/Q_M(t)dt \right) \\ & \cdot \int_{t_1}^{t_2} [E_A(t) - E_M(t)] \cdot C_A(t)/Q_M(t)dt \end{aligned} \tag{A19}$$

Solving for PB gives

$$\mathrm{PB} = \frac{[E_M(t_2) - E_M(t_1)] \cdot \int_{t_2}^{t_3} [E_A(t) - E_M(t)] \cdot C_A(t)/Q_M(t)dt - [E_M(t_3) - E_M(t_2)] \cdot \int_{t_1}^{t_2} [E_A(t) - E_M(t)] \cdot C_A(t)/Q_M(t)dt}{\left(\int_{t_2}^{t_3} E_M(t)/Q_M(t)dt \right) \cdot \left(\int_{t_1}^{t_2} [E_A(t) - E_M(t)] \cdot C_A(t)/Q_M(t)dt \right) - \left(\int_{t_1}^{t_2} E_M(t)/Q_M(t)dt \right) \cdot \left(\int_{t_2}^{t_3} [E_A(t) - E_M(t)] \cdot C_A(t)/Q_M(t)dt \right)}. \tag{A20}$$

By definition, FBR = PB/T, where T is the pool size of amino acids in bound protein. If $T/Q_M(t)$ is defined as $R(t)$, then

$$\mathrm{FBR} = \frac{[E_M(t_2) - E_M(t_1)] \cdot \int_{t_2}^{t_3} [E_A(t) - E_M(t)] \cdot C_A(t)R(t)dt - [E_M(t_3) - E_M(t_2)] \cdot \int_{t_1}^{t_2} [E_A(t) - E_M(t)] \cdot C_A(t)R(t)dt}{\left(\int_{t_2}^{t_3} E_M(t)R(t)dt \right) \cdot \left(\int_{t_1}^{t_2} [E_A(t) - E_M(t)] \cdot C_A(t)R(t)dt \right) - \left(\int_{t_1}^{t_2} E_M(t)R(t)dt \right) \cdot \left(\int_{t_2}^{t_3} [E_A(t) - E_M(t)] \cdot C_A(t)R(t)dt \right)}, \tag{A21}$$

which is the desired formula.

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