Proceedings of the Nutrition Society (2021), **80**, 139–147 doi:10.1017/S0029665120008009

First published online 5 January 2021

Nutrition Society Live 2020 was held virtually on 14–15 July 2020

Symposium two: Novel methods for assessing protein metabolism

Assessing the whole-body protein synthetic response to feeding *in vivo* in human subjects

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All tissues are in a constant state of turnover, with a tightly controlled regulation of protein synthesis and breakdown rates. Due to the relative ease of sampling skeletal muscle tissue, basal muscle protein synthesis rates and the protein synthetic responses to various anabolic stimuli have been well defined in human subjects. In contrast, only limited data are available on tissue protein synthesis rates in other organs. Several organs such as the brain, liver and pancreas, show substantially higher (basal) protein synthesis rates when compared to skeletal muscle tissue. Such data suggest that these tissues may also possess a high level of plasticity. It remains to be determined whether protein synthesis rates in these tissues can be modulated by external stimuli. Whole-body protein synthesis rates are highly responsive to protein intake. As the contribution of muscle protein synthesis rates to whole-body protein synthesis rates is relatively small considering the large amount of muscle mass, this suggests that other organ tissues may also be responsive to (protein) feeding. Whole-body protein synthesis rates in the fasted or fed state can be quantified by measuring plasma amino acid kinetics, although this requires the production of intrinsically labelled protein. Protein intake requirements to maximise whole-body protein synthesis may also be determined by the indicator amino acid oxidation technique, but the technique does not allow the assessment of actual protein synthesis and breakdown rates. Both approaches have several other methodological and inferential limitations that will be discussed in detail in this paper.

Labelled protein: Anabolic: Protein breakdown: RDA

All living tissues are in a state of constant turnover, with a tightly controlled regulation of protein synthesis and breakdown rates[1,2]. This turnover represents tissue plasticity, allowing damaged tissue proteins to be replaced and renewed and facilitates tissue conditioning. In addition, most tissues can atrophy or hypertrophy over time, as a result of a negative or a positive protein balance (protein synthesis < or > protein breakdown, respectively).

Tissue protein synthesis rates can be quantified using stable isotope methodology, by applying the gold standard precursor product method[3]. The most common approach is to combine a primed continuous infusion of labelled amino acids (the precursors) with sequential biopsy collection to determine the rate of labelled amino acid incorporation into tissue protein (the product). This allows the calculation of the tissue protein fractional synthetic rate (often referred to as FSR).

Abbreviation: IAAO, indicator amino acid oxidation.
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The assessment of tissue protein breakdown rates is more complex, especially in the postprandial state[4]. Consequently, data on tissue protein breakdown rates are relatively scarce.

Tissue protein synthesis rates

Due to the relative ease of tissue sampling, much research has been performed on protein metabolism in human skeletal muscle tissue. In a fasted state, muscle protein breakdown rates exceed muscle protein synthesis rates, resulting in a net negative protein balance[5,6]. Physical activity increases muscle protein synthesis rates, and to a lesser extent, muscle protein breakdown rates. However, muscle protein net balance remains negative in the absence of protein ingestion. Protein ingestion increases muscle protein synthesis rates, allowing net muscle protein balance to become positive[7]. Protein or carbohydrate ingestion attenuates muscle protein breakdown rates, mainly via the postprandial rise in circulating insulin levels[8]. Exercise increases basal muscle protein synthesis rates with a stimulation that can persist for as long as 72 h[9]. Furthermore, the muscle protein synthetic response to feeding is (further) increased by prior exercise, with greater muscle protein synthetic responses being observed up to about 24 h after cessation of exercise[10,11]. Consequently, a combination of exercise training and regular protein ingestion will maximise muscle protein synthesis rates, attenuate the exercise-induced increase in muscle protein breakdown and, as such, maximise net muscle protein balance[12].

Although biopsy collection using the percutaneous Bergstrom biopsy approach[13] is frequently applied in skeletal muscle tissue, such biopsies cannot be routinely performed in other tissues. To circumvent the problem of sample collection from various organs, tissue samples can be collected during planned surgery procedures. Using this approach, we have recently assessed basal tissue protein synthesis rates of tendon, ligament, cartilage, bone, liver, pancreas, pancreatic tumour and even brain *in vivo* in human subjects (Fig. 1)[1,14,15]. Basal, post-absorptive tendon, bone and cartilage tissue protein synthesis rates did not differ much from protein synthesis rates in skeletal muscle (about 0·04 %/h). In contrast, protein synthesis rates in liver, pancreas, tumour and brain tissue were several fold (ten to seventeen times) higher when compared to muscle tissue protein synthesis rates. These data prove that such tissues have a high turnover rate and, as such, may express a high level of tissue plasticity.

It has been well-established that protein ingestion stimulates muscle protein synthesis rates *in vivo* in human subjects. It remains to be determined whether protein ingestion also affects tissue protein synthesis rates in other organs. Some indirect evidence suggests that it can be derived from the observation that whole-body protein synthesis rates are highly responsive to protein ingestion[16–18]. Although muscle tissue contributes as much as about 40 % to the whole-body protein pool, it is estimated that it contributes only about 25 % to (post-absorptive) whole-body protein synthesis[19]. Therefore, the large whole-body protein synthetic response that is observed following protein ingestion may be, at least partly, attributed to other tissues that increase their turnover. This is further supported by observed increases in whole-body protein synthesis rates in the absence of a concomitant increase in muscle protein synthesis rates[20,21].

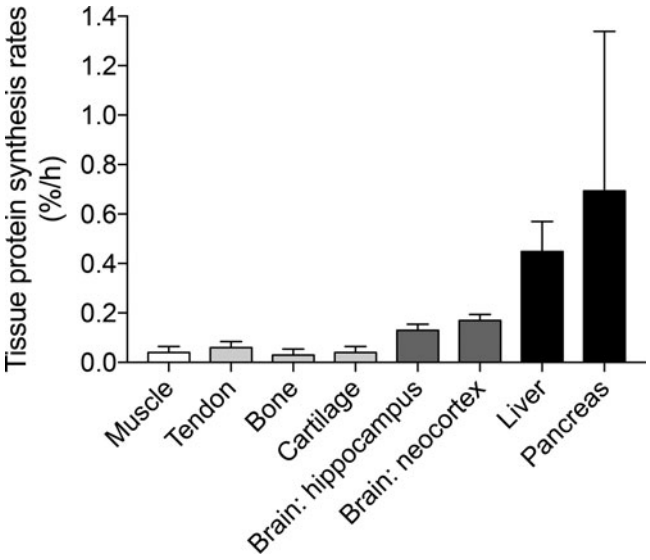

Fig. 1. Postabsorptive protein synthesis rates in various tissues. Data represent means(SD) and are adapted from[1,14,15].

In this review, we will describe the assessment of post-prandial whole-body protein metabolism based on the plasma amino acid kinetics method and the indicator amino acid oxidation (IAAO) technique. In addition, we will critically assess their strengths, weaknesses and discuss practical limitations.

Plasma amino acid kinetics method

Whole-body protein metabolism can be assessed based on plasma amino acid kinetics, i.e. the rates at which amino acids appear and disappear from the circulation (Fig. 2). In the basal, post-absorptive state, protein breakdown is the only process responsible for the release of amino acids into the circulation. Therefore, the amino acid rate of appearance represents the whole-body protein breakdown rate. In contrast, the rate of amino acid disappearance from the circulation represents amino acid uptake into tissues. Following uptake in tissues, amino acids are assumed to have two metabolic fates, either oxidation or incorporation into tissue protein (i.e. protein synthesis). Amino acid oxidation can be measured by quantifying the irreversible hydroxylation of phenylalanine to tyrosine. Subsequently, the protein synthesis rate can be calculated by subtracting the rate of amino acid oxidation from the rate of amino acid disappearance. Finally, protein balance can be assessed by

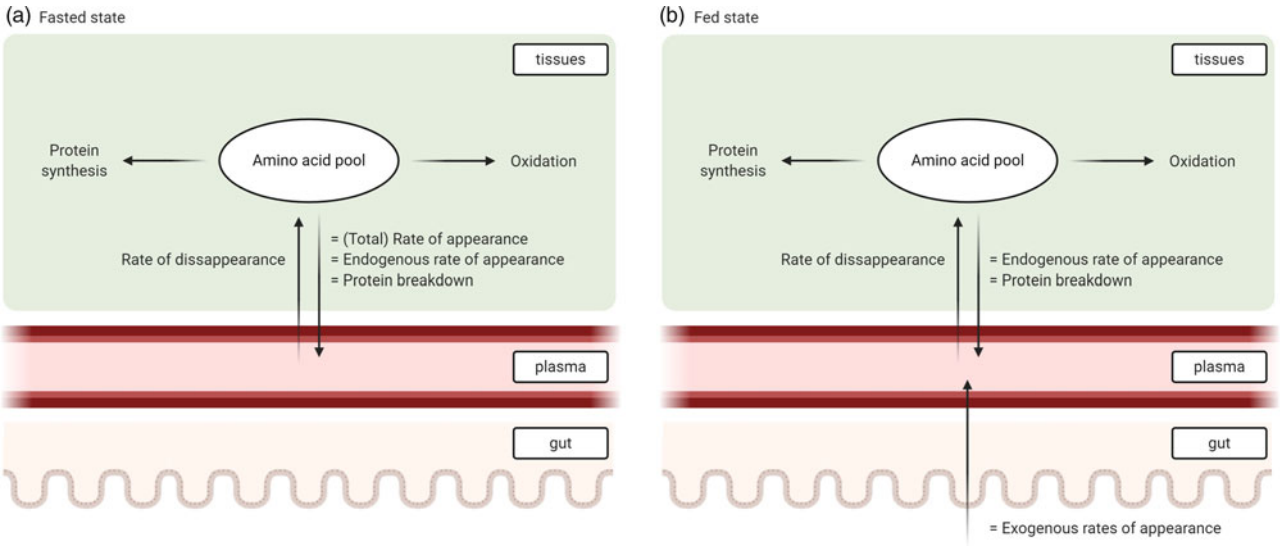

Fig. 2. Schematic representation of plasma amino acid kinetics in the basal and postprandial states.

subtracting protein breakdown rate from whole-body protein synthesis rates. In the fed state, the same concept applies with the exception that (total) amino acid appearance rate is composed of an endogenous component that reflects protein breakdown rate, but also of an exogenous component that reflects amino acids appearing in the circulation as a result of amino acid or protein feeding. By quantifying the exogenous rate of amino acid appearance and subtracting it from the total rate of appearance, the endogenous amino acid rate of appearance (reflecting postprandial protein breakdown rates) can be deduced.

The most common approach to quantify postprandial plasma amino acid kinetics is by a triple tracer technique that consists of the constant infusion of (e.g.) L-[$^2H_5$]-phenylalanine and L-[ring-$^2H_2$]-tyrosine, the ingestion of a L-[1-^{13}C]-phenylalanine-labelled protein bolus and frequent plasma sampling to assess plasma amino acid concentrations and plasma tracer enrichments (Fig. 3)[16,17,20,21]. Plasma amino acid kinetics (i.e. total rate of disappearance and total, endogenous and exogenous rate of appearance) and whole-body protein metabolism (i.e. protein synthesis, breakdown, oxidation and net balance) can be calculated by using modified Steele's equations[22]:

$$\text{Total } R_a = \frac{F_{phe,iv} - [pV \times C(t) \times \Delta E_{iv}/\Delta t]}{E_{phe,iv}(t)} \quad (1)$$

$$\text{Exo } R_a = \text{Total } R_a \frac{E_p,\text{oral}_{(t)} + [pV \times C(t) \times \Delta E_{p,oral}/\Delta t]}{E_{oral}} \quad (2)$$

$$\text{Phe}_{plasma} = \frac{(\text{AUC Exo } R_a)}{\text{Phe}_{oral}} \times \text{BW} \times 100\% \quad (3)$$

$$\text{Protein breakdown} = \text{Endo } R_a = \text{total } R_a - \text{Exo } R_a - F_{phe,iv} \quad (4)$$

$$\text{Total } R_d = \text{total } R_a - pV \times \frac{\Delta C}{\Delta t} \quad (5)$$

$$\text{Protein oxidation} = \text{Tyr } R_a \frac{E_{D4\,tyr}(t)}{E_{phe,iv}(t)} \frac{R_d}{F_{phe,iv} + R_d} \quad (6)$$

$$\text{Protein synthesis} = \text{Total } R_d - \text{Oxidation} \quad (7)$$

$$\text{Net balance} = \text{Protein synthesis} - \text{protein breakdown} \quad (8)$$

Here, $F_{phe,iv}$ represents the intravenous tracer (L-[$^2H_5$]-phenylalanine) infusion rate. pV (0·125 litres/kg) represents the distribution volume of phenylalanine[22]. $C(t)$ represents the mean plasma phenylalanine concentration between two consecutive time points. $\Delta E_{iv}/\Delta t$ represents the time-dependent variation of plasma phenylalanine enrichments derived from the intravenous tracer (L-[$^2H_5$]-phenylalanine). $E_{phe,iv}(t)$ represents the mean plasma phenylalanine enrichment derived from the intravenous tracer (L-[$^2H_5$]-phenylalanine) between two consecutive time points. Exo R_a represents the rate at which dietary protein-derived phenylalanine enters the circulation. $\Delta E_{p,oral}(t)$ represents the mean plasma phenylalanine enrichment derived from the oral tracer (L-[1-^{13}C-phenylalanine]) between two consecutive time points. $\Delta E_{p,oral}/\Delta t$ represents the time dependent variation of the plasma phenylalanine enrichments derived from the oral tracer oral (L-[1-^{13}C-phenylalanine]). E_{oral} represents the (L-[1-^{13}C-phenylalanine]) enrichment of the dietary protein. Phe_{plasma} represents the percentage of dietary protein-derived phenylalanine appearing in the circulation. AUC Exo R_a represents the area under the curve of Exo R_a, which corresponds to the amount of dietary protein-derived phenylalanine that appeared in the circulation throughout the postprandial assessment period. Phe_{oral} represents the amount of phenylalanine ingested. BW is the participants' bodyweight. Total R_d is the total rate of phenylalanine disappearance and represents the rate of phenylalanine hydroxylation (first step in phenylalanine oxidation) plus the rate of phenylalanine utilisation

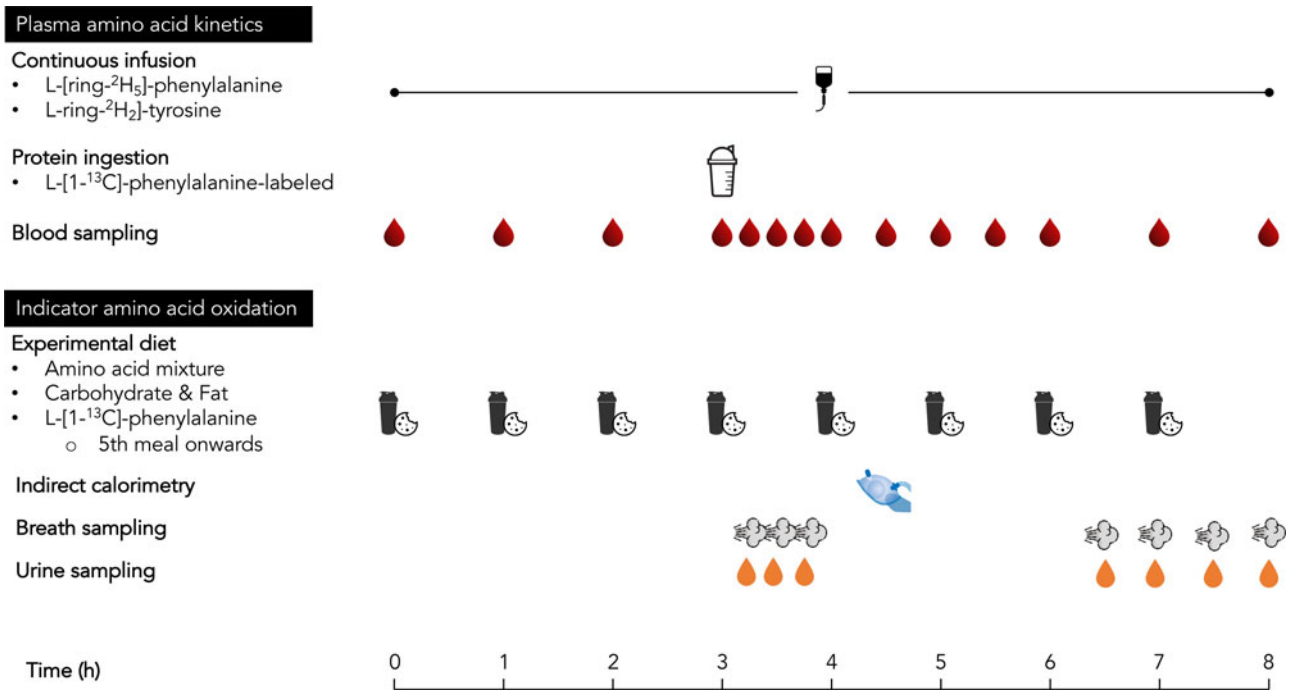

Fig. 3. Schematic representation of the general plasma amino acid kinetics method and indicator amino acid oxidation experimental trials.

for protein synthesis. Tyr R_a represents the total rate of tyrosine appearance based on L-[ring-$^2H_2$]-tyrosine infusion rate and plasma enrichments. $E_{D4tyr}(t)$ represents the mean plasma L-[ring-$^2H_4$]-tyrosine enrichments between two consecutive time points. $E_{phe,iv}(t)$ represents the mean plasma L-[ring-$^2H_5$]-phenylalanine enrichment between two consecutive time points.

Indicator amino acid oxidation technique

The main applications of the IAAO technique are the assessment of protein requirements or the requirements of a single indispensable amino acid[23]. The IAAO technique is based on the concept that all indispensable amino acids need to be available in sufficient quantity to allow protein synthesis. When one or more indispensable amino acids are insufficiently available (i.e. limiting), other available amino acids are in excess and will be oxidised. The technique involves providing an indicator indispensable amino acid (typically ^{13}C-labelled phenylalanine) to quantify amino acid oxidation based on breath analyses (i.e. the rate of $^{13}CO_2$ production). To assess protein requirements, multiple metabolic trials are performed during which the indicator amino acid is provided at the same (excess) amount, but the other amino acids are provided in graded amounts during the different trials (Fig. 4). When amino acids are provided at a low dose, the availability of one or more indispensable amino acids will be limiting for protein synthesis. Consequently, the large excess of the indicator amino acid will result in a high (indicator) amino acid oxidation. As protein intake levels increase, the excess and thereby the oxidation of the indicator amino acid decreases. Once the amino acid requirements of all indispensable amino acids are met, oxidation of the indicator amino acid will become minimal and will remain minimal even when the intake of other amino acids is further increased. The amino acid intake level at which amino acid oxidation becomes minimal (termed the breakpoint) represents the intake level that maximises whole-body protein synthesis rates. Therefore, the breakpoint represents the estimated average requirement to optimise whole-body protein synthesis rates. The upper 95 % CI of the estimated average requirement represents the RDA. The same concepts apply for the assessment of indispensable amino acid requirements, except that graded amounts of the indispensable amino acids are provided while all other amino acids are provided in excess.

The most common application of the IAAO technique is to provide an oral amino acid mixture (among other nutrients) in an hourly sip-feeding protocol to establish steady-state conditions (Fig. 3)[24–26]. A within-subject design is used in which subjects typically perform two to seven metabolic trials on different days during each of which a different amino acid intake level is provided. The amino acid mixture is typically modelled after egg protein, with the exception of phenylalanine and tyrosine that are provided in an excessive and constant amount during each trial. From the fifth meal onwards, L-[1-^{13}C]-phenylalanine is included in the amino acid mixture. Indirect calorimetry is performed once, and breath and urine samples are collected at regular time intervals.

In addition to establishing protein requirements, the IAAO technique can also be used to calculate

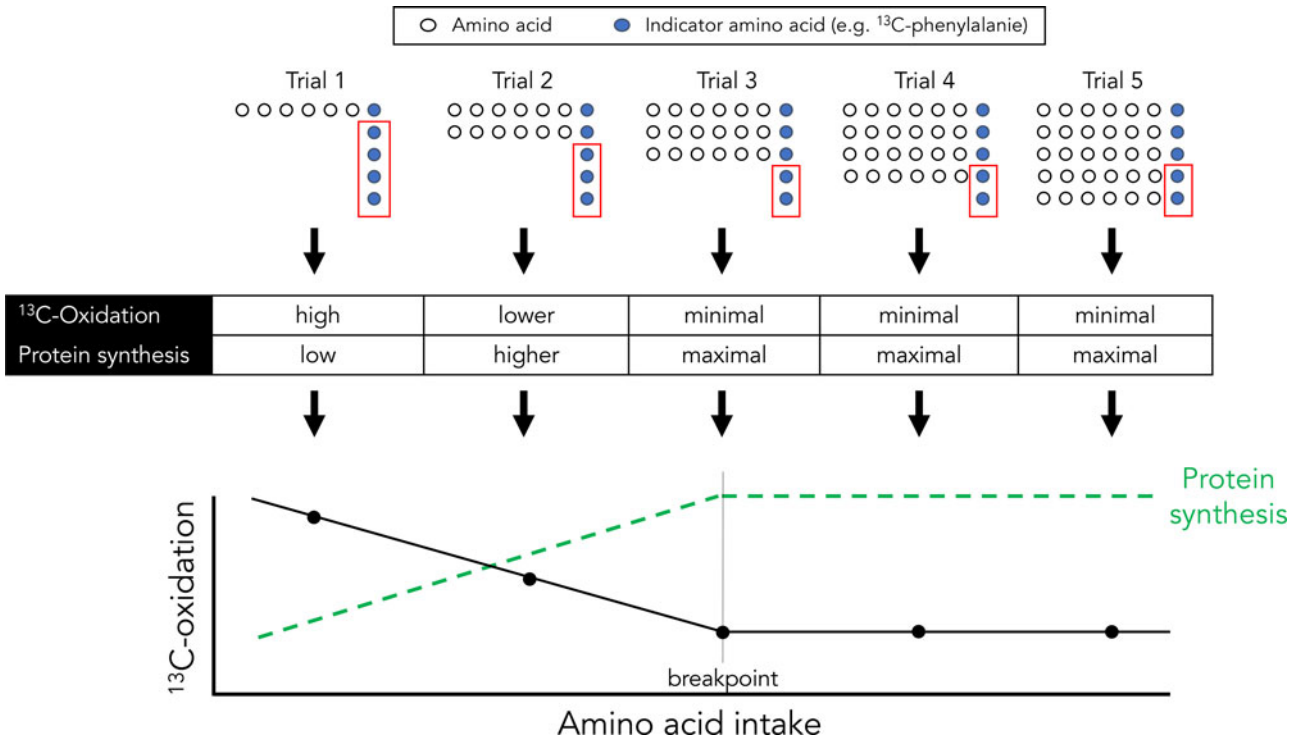

Fig. 4. Schematic representation of the concept and the application of the indicator amino acid oxidation technique to assess the protein intake level that maximises whole-body protein synthesis rates. In a cross-over design, a subject undergoes 5 separate trial days with different amino acid intake levels (trials 1–5). The intake of indicator amino acid is identical during all trials. Trial 1: When amino intake is low, there is a large excess of the indicator amino acid that will be oxidised (red box). Trials 2 and 3: Higher amino acid intake levels allow more of the indicator amino acid to be incorporated into tissues (protein synthesis). Therefore, there is a lower excess and oxidation of the indicator amino acid. Trials 4 and 5: There is a maximal capacity to utilise amino acids for protein synthesis. Increasing amino acid intake beyond the level required to maximise protein synthesis does not result in additional tissue incorporation of the indicator amino acid. Therefore, oxidation of the indicator amino acid plateaus at high amino acid intake levels. Biphasic linear regression is used to identify the breakpoint that represents acid intake level that minimises protein oxidation and maximises protein synthesis.

whole-body protein metabolism[24,25]:

$$\text{Phe } R_a = \text{protein breakdown} = i\frac{E_{oral}}{E_{urine}} - I \quad (9)$$

$$F^{13}CO_2 = V_{CO_2} \times E_{CO_2} \times 44 \cdot 6 \times 60 \times BW^{-1} \times 0 \cdot 82 \times 100\% \quad (10)$$

$$\text{Oxidation} = F^{13}CO_2 \left(\frac{1}{E_{urine}} - \frac{1}{E_{oral}}\right) \times 100 \quad (11)$$

$$\text{Protein synthesis} = \text{Phe } R_a - \text{Oxidation} \quad (12)$$

Here, Phe R_a represents the phenylalanine rate of appearance. The term i represents the rate of [1-^{13}C]-phenylalanine ingestion. E_{oral} represents the phenylalanine enrichment of experimental drink. E_{urine} represents the phenylalanine enrichment of urine. The term I represents the rate of phenylalanine ingestion. $F^{13}CO_2$ represent the rate of $^{13}CO_2$ appearance in breath. V_{CO_2} represents the volume of CO_2 produced. E_{CO_2} is the breath $^{13}CO_2$ enrichment. 44·6 μmol/kg/h and 60 min/h are constants used to convert $F^{13}CO_2$ to μmol/h. 0·82 represents a correction factor for the amount of CO_2 retained in the bicarbonate pool in the fed state[27]. Net balance is calculated using formula (8).

Formula (9) is conceptually similar to formula (1). Steele's equation is not included, because the IAAO technique is applied during steady state conditions. Instead of an intravenous tracer infusion rate ($F_{phe,iv}$), a tracer ingesting rate (i) is used to calculate Phe R_a. However, not all ingested tracer appears in the circulation due to splanchnic extraction[28]. Therefore, the term i overestimates the amount of L-[1-^{13}C]-appearing in the circulation. Consequently, Phe R_a is overestimated. In addition, formula (9) calculates endogenous (as opposed to total) Phe R_a, as the term I (representing exogenous Phe R_a) is subtracted. Formula (9) is conceptually similar to formula (4) where Exo R_a is subtracted from the Total R_a to obtain Endo R_a (presenting protein breakdown). Finally, the term I should also be corrected for splanchnic extraction. Therefore, we propose modifications to the conventional formulas:

$$\text{Total Phe } R_a = \text{Exo } R_{ai}\frac{E_{oral}}{E_{urine}} \quad (13)$$

$$\text{Endo Phe } R_a = \text{protein breakdown} = \text{Exo } R_{ai}\frac{E_{oral}}{E_{urine}} - \text{Exo } R_{aI} \quad (14)$$

The term Exo R_{ai} represents the rate of the oral ([1-^{13}C]-phenylalanine) tracer appearing in the circulation. The term Exo R_{aI} represents the rate of ingested phenylalanine appearing in the circulation.

Limitations

The plasma amino acids kinetics method and the IAAO technique each has several methodological advantages and limitations. It should be noted that both methods have some degree of flexibility, e.g. they can be modified to minimise the impact of some of their limitations. When comparing the most common applications of these methods, the main advantages of the plasma amino acid kinetics method over the IAAO technique is that it can be used for protein ingestion (as opposed to an amino acid mixture), can be used for bolus feeding (as opposed to sip feeding) and allows accurate assessment of postprandial protein synthesis, breakdown and net balance rates. The main advantages of the IAAO technique over the plasma amino acid kinetics method is that it only requires breath and urine sampling and, as such, is minimally invasive (as opposed to requiring a continuous infusion and frequent blood sampling) and allows for a within-subject design with two to seven trials for each subject (as opposed to a between-subject design).

Both the plasma amino acid kinetics and the IAAO technique assume the tissue-free amino acid pools remain constant. This may be the case during the steady-state conditions in the IAAO technique. In contrast, the tissue-free amino acid pools will show transient expansion following the ingestion of a protein bolus[29]. Such expansion violates the assumption of formula (3) (that protein synthesis and oxidation are the only two metabolic fates of amino acids taken up by tissues), and can result in an overestimation of postprandial protein synthesis rates as assessed with the plasma amino acid kinetics method. This issue can be limited by applying a postprandial assessment period that is long enough to allow the free tissue amino acid pool to return to baseline values. However, even when the tissue-free amino acid pool remains constant, intracellular amino acid (re)cycling may occur[30]. Some of the amino acids released from protein breakdown may be directly reincorporated into protein (utilised for protein synthesis) within a single muscle fibre or tissue cell. Such intracellular amino acid kinetics would not impact plasma kinetics. Therefore, protein synthesis and breakdown rates may be equally underestimated based on plasma amino acid kinetics, with little impact on protein balance.

The plasma amino acid kinetics method determines protein metabolism entirely based on the amino acid flux into and out of the circulation. However, it does not account for amino acid flux outside of the circulation. For example, some dietary-derived amino acids may be incorporated into splanchnic tissues upon first pass[31]. As this flux occurs outside of plasma, it is not accounted for in the plasma amino acid kinetics method. In addition, there likely is some endogenous gut protein loss that is excreted via the faeces[32]. Such flux is not accounted for and results in an underestimation of protein breakdown rates in both the plasma amino acid kinetics method and the IAAO technique.

The IAAO technique applies a sip-feeding protocol to establish steady state conditions. However, a limitation of this sip-feeding approach is that is does not reflect traditional food patterns (e.g. three main meals daily). The muscle protein synthetic response to protein sip feeding has been shown to be attenuated when compared to the ingestion of a single bolus[33]. Therefore, protein requirements determined by the IAAO technique may be overestimated. In addition, the IAAO technique estimates protein requirements based on the provision of a free amino acid mixture. However, the metabolic response to protein ingestion and free amino acids is not necessarily the same. For example, free amino acids are assumed to be 100 % absorbed, while the absorbability and rate of absorption of dietary protein is limited by its digestibility[34]. Furthermore, the IAAO technique has been applied to calculate whole-body protein metabolism. As previously discussed, such calculations require the assessment of the exogenous rate of appearance of labelled and unlabelled phenylalanine. However, the assessment of exogenous rates of appearance requires an oral-intravenous dual tracer approach that is not applied during the IAAO technique[35]. Exogenous rates of appearance are sometimes estimated based on literature values[36]. However, exogenous rates of amino acid appearance are highly dependent on experimental context such as the dose of ingested amino acids, nutrient co-ingestion, (prior) exercise, age and the presence or absence of disease[37–41]. Any deviation from the experimental conditions under literature values for the exogenous rate of appearance were established will introduce error in the estimation. Therefore, the IAAO technique should not be used to calculate whole-body protein metabolism when the exogenous rate of appearance has not been assessed.

Practical inferences

The assessment of postprandial whole-body protein metabolism allows for a holistic view of the anabolic response to feeding. If whole-body protein balance is net negative over a prolonged period, it will result in an undesirable loss of body protein. Conversely, a prolonged net positive whole-body protein balance will result in an increase of body protein over time. An increase in body protein is generally considered beneficial, as it represents an increased protein reserve during potential catabolic conditions[42]. However, organ hypertrophy can also be pathological in nature[43,44]. There is a need to gain more insight into postprandial protein metabolism in different tissues and their consequences. Many currently unanswered questions make practical inferences based on whole-body protein metabolism difficult. For example, what is the contribution of each organ to postabsorptive and postprandial protein metabolism?

Does feeding stimulate a protein synthetic response in various organs? Does a prolonged whole-body protein balance translate into organ hypertrophy? Does an increased organ net protein balance result in improved function or rather dysfunction? Does the (speculated) postprandial protein synthetic response to feeding differ in magnitude between the various organs? Do different organs require different amounts of nutrient intake to optimise their conditioning?

For skeletal muscle, it is clear that the ingestion of about 20 g protein induces a near-maximal muscle protein synthetic response both at rest and during post-exercise recovery in healthy, young adults[12,45]. In addition, protein supplementation augments training-induced gains in muscle mass and strength[46]. Because muscle protein metabolism is well-understood and allows for clear practical inferences, it is often the basis of protein recommendations for populations where muscle mass and function are of high relevance (e.g. athletes or older adults)[47,48]. Sometimes whole-body protein metabolism is suggested as a proxy for muscle metabolism[49]. However, local changes in protein metabolism in skeletal muscle tissue do not seem to correlate well with changes in whole-body protein metabolism[12,16,20,21]. For example, muscle protein synthetic responses to resistance exercise are often observed without a concomitant increase in whole-body protein synthesis[20,21]. Conversely, increases in whole-body protein synthesis rates have been reported without a concomitant increase in muscle protein synthesis rates[16]. Such disparities clearly underline that whole-body protein metabolism is not always a good proxy for muscle metabolism.

Conclusions

It has been well-established that protein ingestion stimulates skeletal muscle protein synthesis rates. In contrast, little is known about the postprandial response to feeding in other organs due to the invasiveness of the required tissue sampling. However, postprandial protein metabolism can be assessed at a whole-body level without the necessity for tissue sampling. The plasma amino acid kinetics approach uses an oral-intravenous triple tracer approach to assess postprandial protein metabolism based on the analyses of blood samples collected frequently over time. This allows the assessment of the anabolic response to protein ingestion, but requires the production of intrinsically labelled protein. The non-invasive IAAO technique uses an oral tracer administration approach to assess the protein intake level that maximises whole-body protein synthesis rates based on the release of isotope label in breath. However, the current application of the IAAO technique does not allow calculation of actual whole-body protein synthesis and breakdown rates. In addition, the IAAO technique uses an amino acid sip-feeding approach that does not reflect a traditional daily food pattern. The assessment of postprandial whole-body protein metabolism allows valuable insights into the anabolic response to feeding and exercise. The increase in whole-body protein synthesis to feeding is greater than what is expected based on its stimulatory effect on muscle tissue, which suggests that other organs largely contribute to the observed anabolic response. Although it seems evident that feeding is not only of relevance to protein balance in skeletal muscle tissue, our understanding of the impact of feeding and/or exercise on other tissues remains limited due to its practical limitations. It is questionable whether a prolonged positive whole-body protein balance results in hypertrophy of one or more tissues and whether this would have functional consequences.

Financial Support

J. T. and L. v. L. have received research grants, consulting fees, speaking honoraria or a combination of these for work on postprandial protein metabolism. See the following pages for a full overview of funding: https://www.maastrichtuniversity.nl/jorn.trommelen and https://www.maastrichtuniversity.nl/l.vanloon.

Conflict of Interest

None.

Authorship

J. T. and L. v. L. wrote the manuscript. All authors edited and approved the final version of the manuscript and agree to be accountable for all aspects of the research. The graphical abstract and Fig. 2 are created with BioRender.com.

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