Advances in stable isotope tracer methodology part 2: new thoughts about an "old" method—measurement of whole body protein synthesis and breakdown in the fed state

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ABSTRACT

Whole-body protein turnover (protein synthesis, breakdown, and net balance) model enables quantification of the response to a variety of circumstances, including the response to meal feeding. In the fed state, the whole-body protein turnover model requires taking account of the contribution of absorbed tracee to the observed total appearance of tracee in the peripheral blood (exogenous appearance, Ra_{EXO}). There are different approaches to estimating Ra_{EXO}. The use of an intrinsically labeled dietary protein is based on the overriding assumption that the appearance in the peripheral circulation of a tracer amino acid incorporated into a dietary protein is exactly proportional to the appearance of absorbed tracee. The bioavailability approach is based on the true ileal digestibility of the dietary protein and the irreversible loss of the tracee in the splanchnic bed via hydroxylation of the tracee (phenylalanine). Finally, Ra_{EXO} can be estimated as the increase above the basal rate of appearance of the tracee using traditional tracer dilution methodology. In this paper, we discuss the pros and cons of each approach and conclude that the bioavailability method is the least likely to introduce systematic errors and is therefore the preferable approach.

INTRODUCTION

Tracer-based models often require assumptions made regarding facets of physiologic processes. Measurement of whole-body protein turnover (protein synthesis, breakdown, and net balance) enables quantification of the response to a variety of circumstances, including the response to meal feeding. In the fed state, the whole-body protein turnover model requires taking into account the contribution of absorbed tracee to the observed total appearance of tracee in the peripheral blood (exogenous appearance, Ra_{EXO}). Recently, use of an intrinsically labeled dietary protein, where the plant or animal is given tracer, which is then incorporated into the resulting protein, which is then fed to an animal or human, has been described. The use of an intrinsically labeled dietary protein is based on the overriding assumption that the appearance in the peripheral circulation of a tracer amino acid incorporated into a dietary protein is exactly proportional to the appearance of absorbed tracee. Alternatively, the bioavailability approach, based on the true ileal digestibility of the dietary protein and the irreversible loss of the tracee in the splanchnic bed via hydroxylation of the tracee (ie, phenylalanine), can be used or Ra_{EXO} can be estimated as the increase above the basal rate of appearance of the tracee using traditional tracer dilution methodology. Here, we discuss the pros and cons of each approach. We provide evidence to conclude that the bioavailability method is the least likely to introduce systematic errors and is therefore the preferable approach.

The gain or loss of body protein is determined by the balance between whole-body protein synthesis and breakdown. Quantitative determination of these processes to calculate the net anabolic response to nutrient consumption using stable isotope tracers dates back to the 1930s.[1] Whole-body protein turnover methodology remains viable for a number of reasons—most importantly, nutrients are consumed at the whole-body level, such that the whole-body protein response to nutrient intake is highly relevant to develop dietary recommendations. Recent advances in protein nutrition have focused on muscle protein metabolism, but muscle protein accounts for less than half of whole-body protein metabolism. Further, the muscle protein synthesis rate can be determined simultaneously with whole-body protein metabolism. Finally, whole-body protein turnover methodology enables the simultaneous determination of rates of protein synthesis and breakdown, and recent studies have highlighted the previously underappreciated role of protein breakdown in the anabolic response to protein intake.[2]

The essential amino acid (EAA) model of protein kinetics calculates whole-body protein breakdown in the postabsorptive state as the rate of appearance (Ra) of the tracee amino acid

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into the free amino acid pool of the body, taking account of the proportional contribution of the tracee to the body protein pool. Most commonly, leucine (Leu) or phenylalanine (Phe) tracers are used. Whole-body protein synthesis in the postabsorptive state is calculated as the difference between the Ra of tracee EAA and the rate of its irreversible disposal, again accounting for the proportional contribution of the tracee to body protein. Following ingestion of a protein meal, the total Ra of tracee (Ra_{TOT}) includes the rate of endogenous tracee released as a consequence of the rate of protein breakdown (Ra_{END}) and the Ra of unlabeled tracee in the peripheral circulation resulting from the digestion and absorption of the tracee EAA in a dietary protein (Ra_{EXO}). In the fed state, the rate of protein breakdown is reflected by the Ra of endogenous tracee (Ra_{END}). Ra_{END} is calculated as Ra_{TOT} minus Ra_{EXO}, and the rate of protein synthesis is calculated as Ra_{TOT} minus the rate of irreversible disposal. Thus, the value of Ra_{EXO} is necessary for the calculation of the rate of protein breakdown (Ra_{END}), but not the rate of protein synthesis, as the process of protein synthesis does not distinguish the source of the precursor tracee. As Ra_{EXO} affects only the calculation of the rate of protein breakdown, an inaccurate value of Ra_{EXO} will also affect the calculation of the net balance between rates of protein synthesis and breakdown. The validity of the quantification of Ra_{TOT} and the rate of tracee oxidation or hydroxylation has been considered in detail.[3] In contrast, the accuracy of the determination of Ra_{EXO}, and the impact of potential errors in its estimation, has received little attention. We will discuss the advantages and limitations of different approaches to quantifying Ra_{EXO}.

Use of intrinsically labeled proteins for quantifying Ra_{EXO}

Intrinsically labeled proteins have been used to quantify the extent of digestion, absorption, and first-pass clearance in the splanchnic bed of dietary proteins for 50 years.[4] The use of intrinsically labeled proteins is elegant in its simplicity. A protein is produced containing the same tracer amino acid as is infused (eg, Leu), but labeled differently. Ra_{EXO} is calculated from its Ra in peripheral blood of tracer from the intrinsically labeled protein. In this paper, we will focus on the use of intrinsically labeled proteins for human studies and therefore consider only the use of stable isotope-labeled tracers.

There are major assumptions underlying the use of intrinsically labeled proteins to quantify Ra_{EXO}. We have recently analyzed the validity of these assumptions and modeled quantitatively the magnitude of potential systematic errors resulting from the use of intrinsically labeled proteins to determine Ra_{EXO}.[4]

The intrinsically labeled protein approach assumes that there is no dilution of ingested tracer in the gastrointestinal tract (GIT) that is not directly reflected in the amount of tracee appearing in the peripheral circulation. We estimate that the intrinsic label is diluted approximately 25% in the GIT as a result of the digestion of endogenous (of body origin) proteins, such as digestive enzymes, mucus and cells that enter the GIT during the digestion of a protein meal.[5] These amino acids are mostly reincorporated (without distinction between tracer and tracee) into GIT proteins, thereby approximately maintaining GIT protein balance. Thus, the dilution of the ingested tracer in the GIT does not correspond to net protein balance, or the appearance of exogenous tracee in peripheral blood.

The intrinsically labeled protein approach also assumes that the splanchnic clearance of absorbed tracer accurately reflects the clearance of unlabeled tracee. However, this is not the case. The process of protein turnover in the splanchnic bed reduces the enrichment of absorbed tracee, even in the absence of net uptake. The reduction of enrichment in absorbed labeled amino acid occurs because the rate of incorporation of tracer into protein (ie, protein synthesis) exceeds the rate of release of labeled amino acid as a consequence of protein breakdown until a plateau enrichment of the splanchnic protein is achieved[2] and this process requires many hours. As in the case of the GIT, the dilution of the tracer as a result of splanchnic protein turnover has no necessary relation to the amount of exogenous tracee appearing in the peripheral blood. The result of the dilution of enrichment of intrinsically labeled protein as described above is an underestimation of Ra_{EXO}, with a resulting overestimation of the rate of protein breakdown.[5]

Finally, it is assumed that Ra_{TOT} can be accurately calculated by the Steele equation[6] and that the rearrangement of the Steele equation used to calculate Ra_{EXO} is valid.[7] The validity of the Steele equation to calculate Ra_{TOT} by tracer dilution has been assessed extensively for a number of tracers, and it is widely accepted that accurate values can be obtained in circumstances in which changes in enrichment over time are modest.[8] On the other hand, the rearrangement of the Steele equation to calculate Ra_{EXO} assumes that there is no recycling of absorbed tracer, but this is known to be untrue. Recycling of tracer results in the calculation of Ra_{EXO} well after all intrinsically labeled tracee has been absorbed,[9] thereby overestimating Ra_{EXO}.

Because of systematic errors occurring in opposing directions, it is difficult to estimate the overall error involved in using an intrinsically labeled protein to quantify Ra_{EXO}. However, our error analysis indicates that Ra_{EXO} is generally underestimated, meaning that rate of protein breakdown in response to protein intake is overestimated.[10] As a result, it has been concluded in experiments using intrinsically labeled proteins that protein breakdown is not suppressed by dietary protein intake (eg,[7]), whereas many studies using a variety of approaches have concluded that suppression of protein breakdown is a key component of the anabolic response to dietary protein or amino acid intake.[2 11–13]

The bioavailability approach for quantifying total appearance of exogenous tracee in peripheral blood

The above discussion highlights the shortcomings of the use of intrinsically-labeled proteins to determine Ra_{EXO}. Estimating the bioavailability of dietary protein is an alternative approach. Bioavailability, as defined here, is calculated from the amount of tracee amino acid in dietary protein consumed, the true ileal digestibility of the tracee in the dietary protein and an estimate of the amount of the absorbed tracee that is cleared by the splanchnic bed without reaching the peripheral circulation. Bioavailability is expressed as the total amount of consumed tracee that appears in the peripheral circulation; a minute-by-minute

determination of Ra_{TOT} is not possible when the bioavailability approach is used. Next, we discuss each of the factors involved in the estimation of bioavailability.

1. *Amount of tracee absorbed.* The amount of dietary protein ingested and the tracee content of the ingested protein are known. True (corrected for gut endogenous amino acids) ileal digestibility can be determined experimentally, but more commonly, it can be estimated from literature values. Although abundant true ileal digestibility data are not available for humans, the pig has proven to be an excellent model (second choice), and if neither human nor pig data are available, values from the rat can be used (third choice).[14] The variability associated with true ileal digestibility (5%)[15] does not greatly impact interpretation of results.
2. *Clearance of absorbed tracee by splanchnic bed.* There are two separate mechanisms by which absorbed tracee can be cleared in the splanchnic bed: net uptake to support splanchnic protein turnover, and irreversible catabolism (hydroxylation in the case of Phe).
3. *Net uptake of absorbed tracee by splanchnic protein turnover.* Absorbed tracee can be cleared in the splanchnic bed by net uptake and incorporated into protein. If it is assumed that the rate of protein synthesis balances the rate of protein breakdown in the splanchnic bed during the absorption of exogenous tracee (ie, no net uptake), then the process of splanchnic protein turnover will not affect the amount of absorbed tracee that reaches the peripheral circulation.

The response of splanchnic protein synthesis and breakdown during amino acid absorption is not known. Dietary protein consumption has been reported to stimulate,[16] not affect[17] or decrease[18] GIT protein synthesis in human subjects. No studies have been published in which the response of liver protein metabolism to feeding has been determined in human subjects, but it was shown that liver protein synthesis in rats is not affected by feeding.[19] It is therefore reasonable to assume that splanchnic protein synthesis and breakdown are not altered acutely by dietary protein consumption. This assumption is reasonablebecause of the equivocal results in the literatureand because the error would be small even if splanchnic protein balance increased after a protein meal. On the basis of the size and turnover rate of the GIT, an increase in net protein synthesis proportionate to the increase in whole-body protein synthesis in response to dietary protein intake would account for less than3%of a 20 gdose of dietary protein.[4] A similar approximation applies to hepatic protein synthesis.[20] Suppression in the breakdown of splanchnic proteins would also cause an error of3%–5%in the calculated value of exogenous appearance. Although any error caused by the failure to account for net uptake of absorbed tracee into splanchnic protein would thus likely be small, such an error would result in the systematic overestimation of bioavailability.

4. *Splanchnic irreversible hydroxylation of absorbed tracee.*The bioavailability approach is particularly suited to the Phe/Tyr model of protein kinetics because the fraction of absorbed tracee irreversibly catabolized in the splanchnic bed can be directly measured. Irreversible loss of tracee is determined as the rate of hydroxylation of Phe to Tyr,[2]and although the measurement of this rate is made at the whole-body level, the reaction occurs exclusively in the liver.[21]The amount of absorbed Phe that is irreversibly hydroxylated in the liver can be calculated as the difference between the measured amount of hydroxylation following Phe ingestion (area under curve over time) minus the amount of Phe hydroxylation measured during the fasted state. $Ra_{EXO}$is calculated as:(total Phe ingested× (true ileal digestibility))minus hydroxylation of absorbed Phe.

Calculation of the rate of hydroxylation of Phe requires dividing the rate of incorporation of label from Phe into Tyr by the precursor (Phe) enrichment, which is the intrahepatic enrichment of Phe.[22] Intrahepatic Phe enrichment cannot be measured in humans, but the relation between plasma and intrahepatic Phe enrichment during a constant tracer infusion is directly reflected by the plateau enrichment of any rapidly turning-over plasma protein that is produced in the liver. Apolipoprotein B-100 is such a protein.[23] During a continuous tracer infusion in the fasted state, the peripheral plasma enrichment of Phe was not different from the intrahepatic precursor enrichment.[23] This observation is consistent with the rapid transmembrane transport of Phe.[24] In the fed state, the intrahepatic enrichment of Phe was 19% lower than the peripheral plasma enrichment.[23] The higher plasma Phe enrichment relative to the true plateau intrahepatic enrichment in the fed state reflects the fact that the absorbed Phe in the portal vein perfusing the liver has not yet had a chance to mix with the Phe tracer in peripheral blood. Since the precursor enrichment is in the denominator of the equation to calculate hydroxylation,[2] use of the plasma enrichment as the precursor results in an underestimation of the rate of hydroxylation of Phe by approximately 20% in the fed (but not the fasted) state. The extent of overestimation of the true intrahepatic enrichment by the peripheral Phe enrichment depends on the amount of dietary food ingested- a large protein meal will result in a larger discrepancy between the portal vein enrichment and the peripheral enrichment.

There are two approaches to taking account of the intrahepatic dilution of the Phe tracer in the fed state. It is possible and preferable to make a direct measurement of the intrahepatic Phe enrichment by determining the plateau enrichment in apolipoprotein B-100. Six to 7 hours of tracer infusion are required to reach a true equilibrium, but 3 hours of tracer infusion is generally sufficient to obtain adequate data to predict the ultimate plateau enrichment by curve fitting.[3] Alternatively, it can be assumed that the true precursor enrichment of Phe in the fed state is approximately 20% lower than the measured peripheral value.

Summary of the bioavailability approach. Literature or determined values can be used to estimate true ileal digestibility. An assumption regarding the extent of net uptake of tracee into splanchnic proteins is required, but errors associated with not making such a correction are likely to be small. The measurement of Phe hydroxylation to Tyr is a direct reflection of the irreversible catabolism of Phe, but account must be taken of the relative reduction in intrahepatic Phe (precursor) enrichment in the fed state. The intrahepatic enrichment can be directly determined as the plateau value of Phe enrichment in apolipoprotein B-100 during a continuous tracer infusion. Alternatively, it can be assumed that the measured peripheral Phe enrichment is a 20% overestimation of the intrahepatic Phe enrichment.

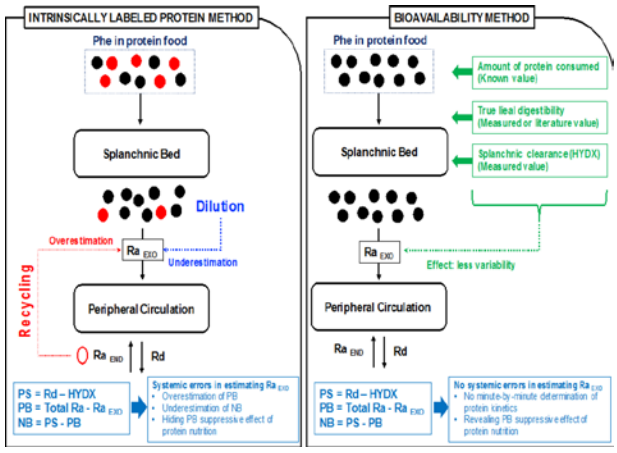

Figure 1 Schematic representations of the intrinsically labeled protein and bioavailability approaches to determining the appearance of exogenous amino acids in the peripheral circulation. The fundamental problem is the determination of the contribution of dietary protein digestion to the total rate of appearance of tracee (ie, from exogenous plus endogenous sources) in order to calculate the rate of protein breakdown. The intrinsically labeled protein approach suffers from systematic errors that are avoided with the bioavailability method. HYDX, hydroxylation; black circle, unlabeled Phe; red circle, labeled Phe; red lined open circle, recycled labeled Phe.

Direct measurement of the appearance of tracee in the peripheral blood

Measurement of Ra_{EXO} can be accomplished by tracer dilution technique using the Steele equation.[6] Subtraction of the fasted Ra_{END} from the measured value of Ra_{TOT} in the fed state will yield Ra_{EXO}. The limitation of this seemingly direct approach is that Ra_{TOT} measured by tracer dilution includes the absorbed Phe and also Phe released as a result of protein breakdown. The Steele equation has been validated in non-steady states in which the changes in enrichment from sample to sample are modest.[25] If the protein meal suppresses protein breakdown, then the Steele equation will underestimate the actual Ra_{EXO}. In a recent series of studies, we determined whole-body protein kinetics in the fasted and fed state in 64 subjects. The bioavailability method indicated that 56.2±1.9 (SEM) per cent of consumed protein appeared in peripheral blood, as compared with the value of 39.1±2.8 (SEM) per cent determined by the measurement of the increase in Ra by the Steele equation. The discrepancy between these two values reflects the extent to which Ra_{TOT} has been reduced as a consequence of suppression of protein breakdown.

CONCLUSION

Figure 1 represents a schematic summary of the intrinsically labeled protein and the bioavailability methods. Use of intrinsically labeled proteins is an innovative approach to determining Ra_{EXO}, but it is limited by the difficulty and expense of producing the labeled proteins. More importantly, the intrinsically labeled protein approach systematically underestimates Ra_{EXO} and thus overestimates the rate of protein breakdown. Direct measurement of Ra_{EXO} by tracer dilution and the Steele equation underestimates the true value by the extent to which protein breakdown is suppressed by the protein meal. The bioavailability approach is limited by the fact that only the total response to dietary protein, rather than a minute-by-minute response, is calculated. The components of the bioavailability approach can be measured, or assumptions can be made based on literature values. Assumptions in the bioavailability approach have inherent errors, but are not likely to result in the systematic overestimation or underestimation of the total appearance of tracee in peripheral blood. The Phe/Tyr model of protein metabolism is optimal when using the bioavailability approach, because irreversible splanchnic hydroxylation of absorbed Phe is measured. When all of the advantages and disadvantages of the possible approaches to estimate the amount of absorbed tracee that enters the peripheral circulation are considered, it is our opinion that the bioavailability approach is preferable to either the intrinsically labeled protein approach or the measurement of Ra_{EXO} by tracer dilution methodology.

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