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Comprehensive assessment of post-prandial protein handling by the application of intrinsically labelled protein *in vivo* in human subjects

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All human tissues are in a constant state of remodelling, regulated by the balance between tissue protein synthesis and breakdown rates. It has been well-established that protein ingestion stimulates skeletal muscle and whole-body protein synthesis. Stable isotope-labelled amino acid methodologies are commonly applied to assess the various aspects of protein metabolism *in vivo* in human subjects. However, to achieve a more comprehensive assessment of post-prandial protein handling *in vivo* in human subjects, intravenous stable isotope-labelled amino acid infusions can be combined with the ingestion of intrinsically labelled protein and the collection of blood and muscle tissue samples. The combined application of ingesting intrinsically labelled protein with continuous intravenous stable isotope-labelled amino acid infusion allows the simultaneous assessment of protein digestion and amino acid absorption kinetics (e.g. release of dietary protein-derived amino acids into the circulation), whole-body protein metabolism (whole-body protein synthesis, breakdown and oxidation rates and net protein balance) and skeletal muscle metabolism (muscle protein fractional synthesis rates and dietary protein-derived amino acid incorporation into muscle protein). The purpose of this review is to provide an overview of the various aspects of post-prandial protein handling and metabolism with a focus on insights obtained from studies that have applied intrinsically labelled protein under a variety of conditions in different populations.

Digestibility: Anabolism: Hypertrophy: Protein quality

All human tissues are in a constant state of remodelling, which is regulated by the balance between tissue protein synthesis and breakdown rates[1,2]. Food ingestion forms a major anabolic stimulus for skeletal muscle tissue[3,4]. Protein ingestion delivers amino acids that stimulate protein synthesis (e.g. leucine) and are used as precursors to support *de novo* protein synthesis. Various dietary factors modulate the protein synthetic response to protein ingestion, including the amount and quality of the ingested protein[5–7]. The capacity of dietary protein to stimulate protein synthesis largely depends on the digestion and amino acid absorption kinetics and amino acid composition of the ingested protein[8,9].

Post-prandial protein handling encompasses the various processes by which the ingested protein is digested, absorbed and metabolised in tissues (e.g. protein synthesis and oxidation). Stable isotope-labelled amino acid methodologies are commonly applied to assess the individual aspects of protein metabolism *in vivo* in human subjects[10–14]. For example, the infusion of stable

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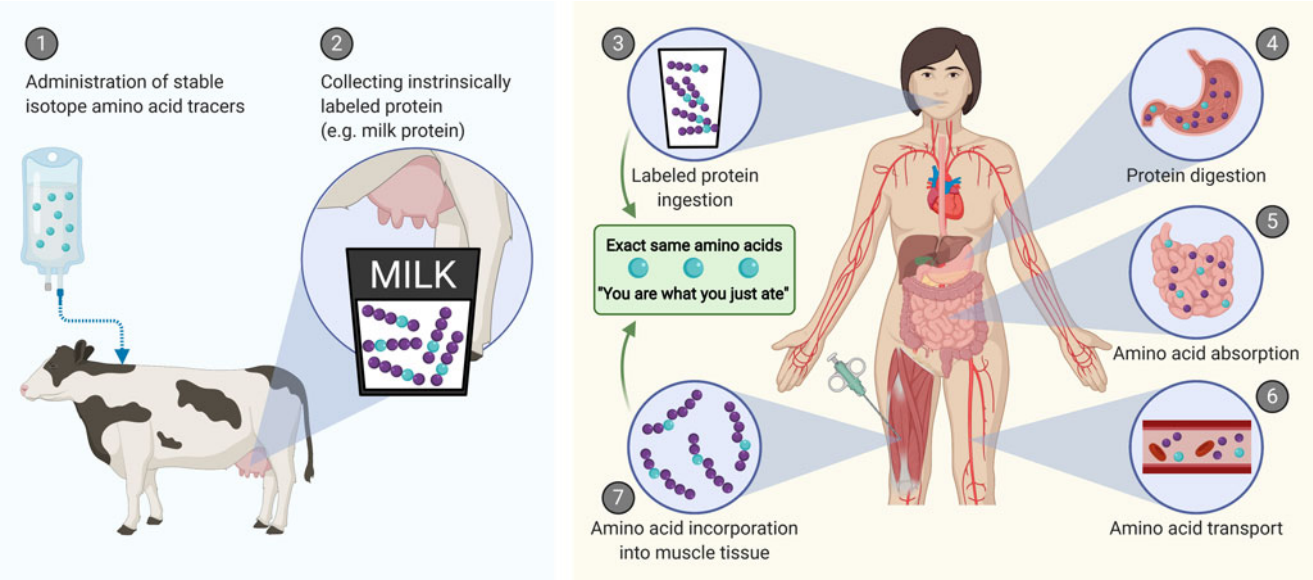

Fig. 1. Schematic representation of the production of intrinsically labelled protein sources to assess various aspects of post-prandial protein handling. Here, the production of intrinsically labelled milk: (1) stable isotope amino acid tracers are administered to lactating cows, (2) the cow produces milk with the amino acid tracer incorporated into the milk protein matrix. Application of intrinsically labelled protein: (3) the collected intrinsically labelled milk protein is consumed by participants, (4) dietary protein is digested into peptides and amino acids, (5) dietary protein-derived amino acids, di- and tri-peptides are taken up from the gastrointestinal lumen by enterocytes, (6) dietary protein-derived amino acids are released into the circulation and (7) dietary protein-derived amino acids are taken up and incorporated into tissues, such as skeletal muscle.

isotope-labelled amino acids is commonly applied to assess basal, post-absorptive or post-prandial muscle protein synthesis rates. However, this approach does not allow the assessment of dietary protein digestion and amino acid absorption kinetics and the subsequent impact on whole-body protein synthesis, breakdown or amino acid oxidation.

A more comprehensive assessment of post-prandial protein handling *in vivo* in human subjects can be achieved by combining the infusion of stable isotope-labelled amino acids with the ingestion of intrinsically labelled protein and frequent collection of blood and muscle samples. Intrinsically labelled milk protein was first obtained by Boirie *et al.* via infusing a lactating cow with L-[1-^{13}C]-leucine, which resulted in the incorporation of L-[1-^{13}C]-leucine into the milk protein matrix[15]. The intrinsically labelled milk was collected and subsequently ingested by human test participants. Blood sample collection and analysis allows the *in vivo* assessment of the post-prandial appearance rate of milk protein-derived L-[1-^{13}C]-leucine into the circulation[16]. We and others have further improved this approach to assess both post-prandial protein handling and subsequent metabolism[17–20]. This approach (Fig. 1) now allows the assessment of (1) protein digestion and amino acid absorption kinetics (total amino acid rate of appearance, protein-derived amino acid rate of appearance, endogenous amino acid rate of appearance and total amino acid rate of disappearance into tissues), (2) whole-body protein metabolism (whole-body protein synthesis, breakdown and oxidation rates and net protein balance) and (3) post-prandial skeletal muscle metabolism (muscle protein fractional synthesis rates and specific dietary protein-derived amino acid incorporation into muscle protein). The purpose of this review is to provide an overview of the various aspects of post-prandial protein handling and metabolism with a focus on insights obtained from studies that have applied intrinsically labelled protein under a variety of conditions in different populations.

Dietary protein-derived amino acid release into the circulation

Dietary protein-derived amino acid release into the circulation is preceded by a series of complex processes, including mastication, gastric mixing, gastric emptying into the intestine, protein cleavage into amino acids, di- and tri-peptides, transport into the enterocytes, amino acid extraction by splanchnic tissues and the release of the remaining dietary protein-derived amino acids into the circulation. The rise in circulating plasma amino acid concentrations is often used as a proxy of dietary protein digestion and amino acid absorption[21–23]. However, changes in plasma amino acid concentrations do not necessarily reflect the mere appearance rate of exogenous (dietary protein-derived) amino acids as they are also impacted by changes in the release of amino acids originating from various tissues and the (rapid) uptake of amino acids in these tissues. The post-prandial rise in circulating plasma amino acid concentrations reflects dietary protein digestion and amino acid absorption as well as the uptake (synthesis and oxidation) and

release (breakdown) of amino acids by other tissues. Therefore, a more direct assessment of the rate of protein-derived exogenous amino acid release into the circulation (EXO_{Ra}) is required to determine to what extent the ingested protein is digested, absorbed and subsequently released as amino acids into the circulation. To assess the EXO_{Ra} *in vivo* in human subjects, an intravenous stable isotope amino acid infusion is combined with the ingestion of intrinsically labelled protein in which the same amino acid with a different isotope label is incorporated within the protein matrix[24]. The labelled dietary protein-derived amino acids are digested and absorbed identically to unlabelled amino acids, allowing us to quantify the EXO_{Ra} of the dietary protein. By calculating the incremental area under the curve of exogenous amino acid rate of appearance, the total amount of dietary protein-derived amino acids appearing in the circulation can be calculated and/or expressed as a percentage of the amount of the specific amino acid provided within the ingested protein.

Factors affecting dietary protein-derived amino acid release into the circulation

The combination of intrinsically labelled protein with intravenous stable isotope amino acid infusion has been applied to identify different dietary factors that modulate EXO_{Ra}. The ingestion of greater amounts of protein results in a greater EXO_{Ra} up to (at least) the ingestion of as much as 45 g protein[6,25–27]. Different types of proteins also modulate EXO_{Ra}. For instance, casein protein ingestion results in a relatively lower but more prolonged EXO_{Ra}, whereas whey protein ingestion results in a relatively rapid, but more transient, increase in EXO_{Ra}[8,16,26]. This difference has been attributed to the clotting of micellar casein protein in the acidic environment of the stomach, thereby delaying gastric emptying and subsequent protein digestion[28]. Milk protein ingestion results in an intermediate EXO_{Ra} when compared to whey and casein protein ingestion[26]. Milk protein ingestion is followed by a lower EXO_{Ra} when compared to the ingestion of an equivalent amount of minced (cooked) beef[29]. The digestion and absorption kinetics of ingested protein are not only dependent on the inherent properties of the protein, but are also modulated by protein processing and/or meal preparation. In this regard, the ingestion of a protein hydrolysate results in an accelerated EXO_{Ra} when compared with the ingestion of the same, intact protein[8,30]. In a similar context, the ingestion of minced (cooked) meat results in a higher EXO_{Ra} when compared to the ingestion of (cooked) beef steak[31]. Furthermore, co-ingestion of other macronutrients impacts the EXO_{Ra} following protein ingestion. For instance, carbohydrate co-ingestion attenuates the EXO_{Ra}[32]. In agreement, the EXO_{Ra} of casein is attenuated when ingested within a carbohydrate-containing milk matrix[33]. However, the co-ingestion of milk fat within a casein protein beverage does not impact EXO_{Ra}[34]. In contrast, egg-white consumption resulted in a more rapid EXO_{Ra} when compared to the ingestion of an isonitrogenous amount of whole egg that contained substantially more fat[35]. The discrepancy in the impact of fat co-ingestion on EXO_{Ra} is possibly due to how fat interacts with protein within the food matrix. For instance, co-ingested fat likely separates from protein within the stomach following consumption as a beverage, whereas fat and proteins may remain homogenous in a matrix following whole egg ingestion, facilitating greater macronutrient interaction[36].

In addition to dietary factors, the EXO_{Ra} is modulated by physiological conditions. Protein ingestion in older individuals results in lower EXO_{Ra} when compared to the ingestion of the same amount of protein by younger adults[26,32,37], and EXO_{Ra} is lower in obese *v.* lean individuals[38]. Furthermore, EXO_{Ra} seems to be attenuated during recovery from resistance-, but not endurance-type exercise[39,40]. Finally, maintenance haemodialysis patients display a lower EXO_{Ra} following egg ingestion when compared to healthy controls[41]. Taken together, it is clear that dietary protein-derived amino acid release is strongly modulated by several factors related to the composition and preparation of the ingested meal along with other physiological factors.

Post-prandial whole-body protein metabolism

The assessment of whole-body protein metabolism (i.e. protein synthesis, breakdown and amino acid oxidation rates) is based on the rates of amino acid appearance in and disappearance from the circulation. In a fasted state, protein breakdown is the only source of amino acid release into the circulation and can be quantified based on a continuous intravenous stable isotope amino acid infusion. In the fed state, amino acids released in the circulation can be from endogenous and exogenous origin as a result of tissue protein breakdown and protein ingestion, respectively. The application of intrinsically labelled protein enables us to differentiate between dietary protein-derived amino acids and endogenous amino acids release. Therefore, the combined application of intravenous stable isotope amino acid infusions with the ingestion of intrinsically labelled protein allows us to accurately assess post-prandial protein handling.

Factors affecting post-prandial whole-body protein metabolism

The combined application of continuous intravenous infusion of stable isotope amino acids and ingestion of intrinsically labelled protein has been used to establish that intrinsically labelled milk protein ingestion stimulates whole-body protein synthesis rates, attenuates whole-body protein breakdown rates and improves whole-body protein net balance in a dose-dependent manner up to doses of at least 45 g[6,25,27]. This response can be modulated by the type of protein ingested. For example, the ingestion of intrinsically labelled minced beef has been shown to improve whole-body net protein balance to a greater extent than ingesting the same beef in the form of an intrinsically labelled steak[31]. Carbohydrate co-ingestion has been shown to further reduce whole-body protein breakdown and amino acid

oxidation[32]. This may in part be attributed to its insulinotropic properties[37].

Apart from nutritional factors, physiological factors also modulate the effect of protein ingestion on whole-body protein metabolism. Resistance- and endurance-type exercises appear to have little-to-no impact on whole-body net protein balance[40,42,43]. The post-prandial reduction in whole-body protein breakdown rates is attenuated in older adults when compared to younger adults[32]. In line, older adults display less of a post-prandial increase in whole-body net balance when compared to younger adults[37]. Finally, obese individuals seem to display less of a post-prandial increase in whole-body net protein balance when compared to lean individuals following ingestion of 25 g protein. The latter may be explained by the lower dosage when expressed per kg body mass, resulting in less of an increase in post-prandial amino acid availability expressed per kg body mass[38]. Although several factors have been shown to impact whole-body net protein balance, post-prandial changes in plasma amino acid availability seem to represent the main determinant defining the impact of feeding on the post-prandial whole-body net protein balance.

Post-prandial muscle protein metabolism

The assessment of post-prandial whole-body protein metabolism allows a holistic view of the anabolic response of the sum of all tissues to anabolic stimuli, such as food intake and physical activity. However, different organs and organ regions have substantially different tissue protein synthesis rates and may respond quite differently to various stimuli[1,44,45]. Therefore, whole-body metabolic responses may not represent the response of a specific tissue of interest. To directly assess protein synthesis rates in a specific tissue, a continuous intravenous infusion of stable isotope amino acids is combined with tissue biopsy sampling, using the precursor-product method[46]. The precursor-product method is most commonly performed to determine fractional protein synthesis rates in skeletal muscle tissue, which represents the organ containing the largest proportion of total body protein due to its extensive mass (40 % of whole-body protein pool). Furthermore, biopsy sampling from skeletal muscle tissue is relatively easy when compared to tissue collection from other organs[47]. Furthermore, skeletal muscle tissue is highly adaptive in response to physical activity and nutrition[5]. Given that the stimulation of skeletal muscle protein synthesis is a key regulatory factor for muscle mass and function, the precursor-product method has been applied extensively to study skeletal muscle metabolism in various populations and conditions. Dietary and physiological factors affecting muscle protein synthesis have been the topic of several recent reviews[5,48,49].

The use of intrinsically labelled protein to quantify post-prandial muscle protein synthesis

Measurement of the muscle protein synthetic response following food (protein) intake is of great importance, but accurate quantification can be challenging. An accurate assessment of muscle protein synthesis requires a steady state in the stable isotope-labelled amino acid precursor pool (i.e. in the plasma or muscle-free amino acid pools). However, protein ingestion is followed by the release of unlabelled amino acids into the circulation, which destabilises the precursor pool(s) by rapid dilution followed by a gradual return to steady-state conditions (Fig. 2a)[50]. Destabilisation of the precursor pools during the post-prandial period may lower the capacity to detect small changes in muscle protein synthesis rates following food intake (Fig. 2b)[50]. However, such a destabilisation in the precursor pools can be prevented by the application of intrinsically labelled protein, in which the labelled amino acid in the protein matches the labelled amino acid that is intravenously infused (Fig. 2c). To avoid precursor destabilisation, the labelled amino acid enrichment in the dietary protein must be comparable to the plasma enrichment that will be reached during intravenous-labelled amino acid infusion. Upon protein ingestion, the dietary protein-derived labelled amino acids are digested and absorbed identically to the unlabelled protein-derived amino acids. As a result, the labelled and unlabelled amino acids are uniformly delivered into the plasma and peripheral tissues, which allows maintenance of a steady-state isotope tracer enrichment of the precursor pool(s). This allows for a more sensitive assessment of the muscle protein synthetic response to feeding (Fig. 2d). Although the use of intrinsically labelled protein forms the preferred approach to avoid precursor pool destabilisation during the measurement of post-prandial muscle protein synthesis rates, the co-ingestion of labelled free amino acids with dietary protein represents a less costly alternative. However, free amino acids are much more rapidly absorbed in comparison with intact protein. Therefore, free amino acid co-ingestion may result in a transient increase in precursor enrichment during the early post-prandial period, before eventually returning to steady-state conditions (Fig. 3a). When labelled free amino acids are co-ingested, more frequent plasma sampling should be applied to confirm and appropriately characterise the destabilisation of the post-prandial isotope tracer precursor pool enrichment (Fig. 3b).

De novo muscle protein synthesis

In addition to facilitating the assessment of post-prandial muscle protein synthesis rates, intrinsically labelled protein allows for the assessment of the incorporation of dietary protein-derived amino acids into muscle tissue protein (i.e. *de novo* muscle protein synthesis; Fig. 2f). This measurement requires the intrinsically labelled protein to be highly enriched with an amino acid label that is different from the one infused intravenously. Intrinsically (doubly) labelled protein containing two different labelled amino acids (one matching the amino acid tracer applied in the infusate) allows the simultaneous assessment of the incorporation of dietary protein-derived amino acids into muscle proteins and post-prandial

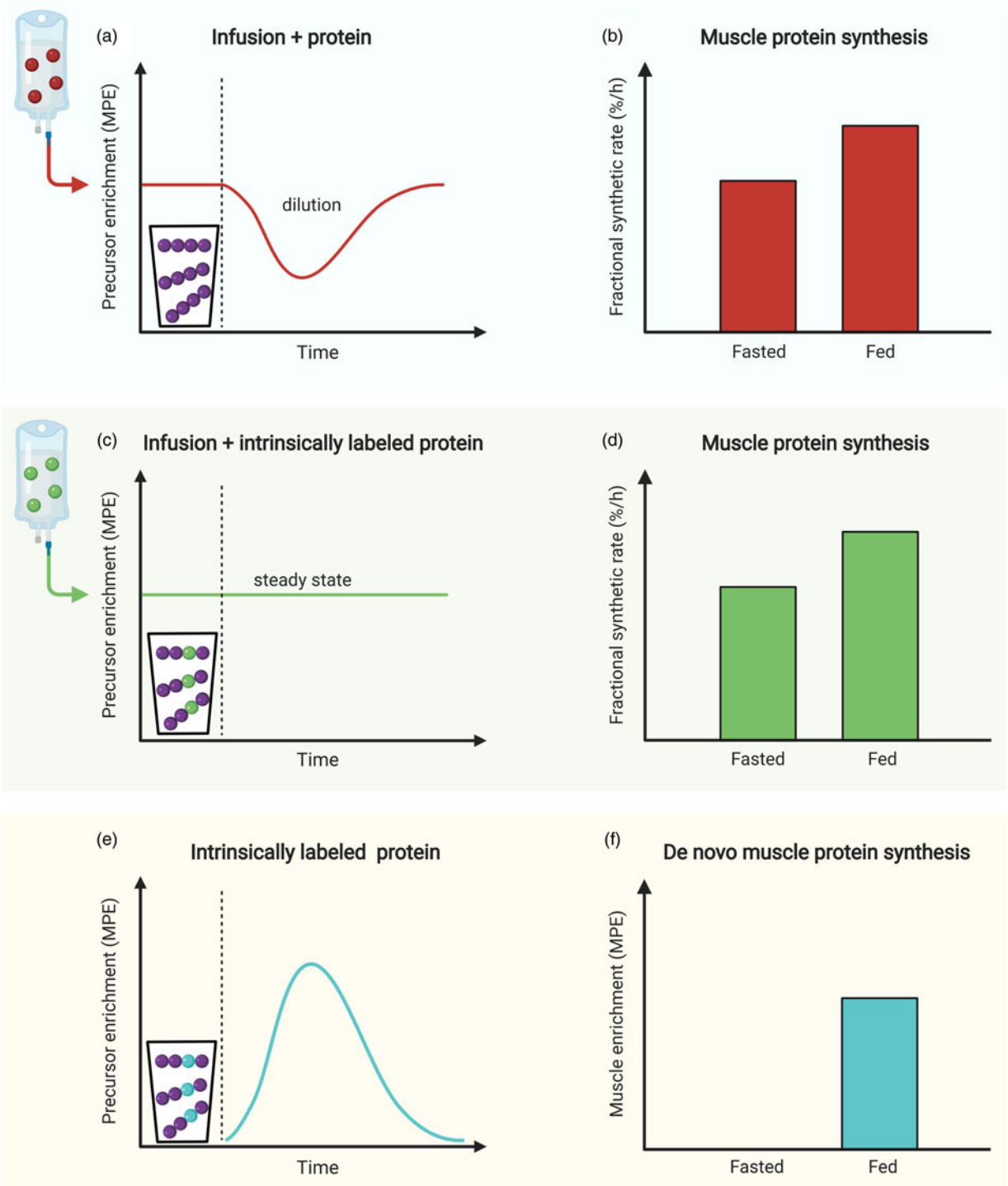

Fig. 2. Schematic representation of the use of intrinsically labelled dietary protein to accurately quantify post-prandial muscle protein synthesis rates as well as *de novo* muscle protein synthesis. (a) and (b): When protein is ingested, the steady-state precursor enrichment obtained by intravenous infusion of stable isotope amino acid tracers is diluted. This dilution of the precursor pool enrichment may compromise the ability to quantify the muscle protein synthetic response to feeding. (c) and (d): When ingesting intrinsically labelled protein, in which the labelled amino acid in the protein matches the labelled amino acid that is intravenously infused, dilution of the precursor pool can be prevented and tracer steady-state may be maintained. This allows for a more accurate measurement of the muscle protein synthetic response to feeding. (e) and (f): The presence of a labelled amino acid (that is not infused) in the dietary protein allows for the assessment of the incorporation of dietary protein-derived amino acids into muscle tissue protein (i.e. *de novo* muscle protein synthesis).

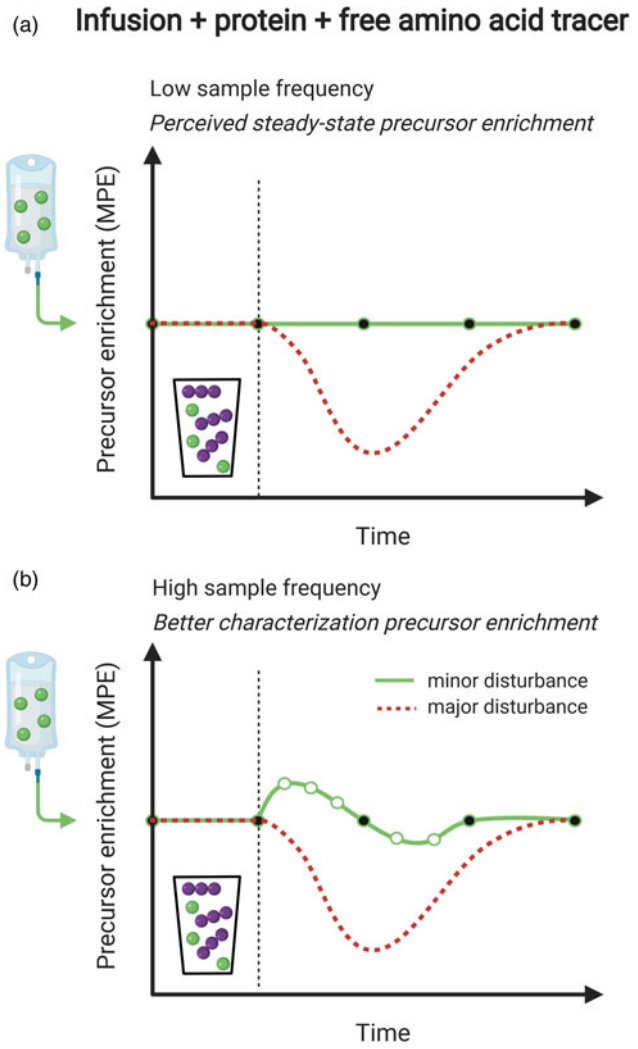

Fig. 3. Schematic representation of the co-ingestion of labelled free amino acids (corresponding to intravenously infused labelled amino acids) with dietary protein as a means to maintain precursor pool enrichments for the accurate measurement of post-prandial muscle protein synthesis rates. (a): The red dotted line represents dilution of the (infused) labelled amino acid precursor pool following the ingestion of dietary protein. Co-ingestion of the same labelled amino acid allows maintenance of isotope tracer steady-state conditions. (b): Ingestion of a free, crystalline (isotope-labelled) amino acid will be more rapidly absorbed when compared to the ingestion of the same (isotope-labelled) amino acid when it is incorporated into an intact protein. Therefore, co-ingestion of a labelled amino acid cannot prevent disruption of the isotope tracer steady-state. Therefore, it is at all times important to correctly assess changes in precursor pool enrichments over time, which requires a high blood or tissue sampling frequency.

fractional muscle protein synthesis rates. The direct measurement of the incorporation of protein-derived amino acids into skeletal muscle protein encompasses all upstream processes of dietary protein handling, including protein digestion and amino acid absorption, delivery to skeletal muscle tissue, transport into the muscle tissue and utilisation as a precursor for *de novo* muscle protein synthesis. As such, the direct assessment of the incorporation of dietary protein-derived amino acids in tissue protein indicates to what extent the ingested dietary protein is delivered to and utilised within in the tissue to support its remodelling. Therefore, highly enriched intrinsically labelled protein can provide valuable information on how dietary protein is utilised by tissues in a variety of experimental settings.

Factors affecting the incorporation of dietary protein-derived amino acids into muscle protein

The use of highly enriched intrinsically labelled protein has consistently shown that dietary protein-derived amino acids are utilised for *de novo* muscle protein synthesis across a wide variety of populations and can be modulated by various dietary and physiological conditions[3,27,51,52]. Ingested protein delivers amino acids as precursors for *de novo* muscle protein synthesis in mixed muscle protein[3], and has also been detected in various isolated muscle protein fractions, such as myofibrillar[51], mitochondrial[6] and connective tissue[53] proteins. Our research group has applied intrinsically labelled protein to demonstrate that the ingestion of 20 g micellar casein protein results in the incorporation of approximately 2 g (about 10 %) dietary protein-derived amino acids into muscle tissue protein during a 5 h post-prandial period[3]. The observation that dietary protein-derived amino acids are directly incorporated into skeletal muscle protein within the hours following meal ingestion provided us with the evidence that 'you are what you just ate'[3]. More recent study has demonstrated that the ingestion of greater amounts of dietary protein results in greater amounts of dietary protein-derived amino acids being incorporated into skeletal muscle protein, with no indication of an upper limit up to the ingestion of a dose of 45 g protein[6,25,27]. However, aside from the amount of ingested protein, the type of ingested protein has also been shown to impact dietary protein-derived amino acid incorporation into skeletal muscle protein. In particular, whey protein has been shown to result in a greater incorporation of dietary protein-derived amino acid into skeletal muscle proteins when compared to micellar casein and hydrolysed micellar casein[8]. This may be explained by the more rapid release of protein-derived amino acids following whey *v.* casein ingestion and the higher leucine content of whey protein[54,55].

Besides dietary factors, physiological factors have also been shown to impact the incorporation of dietary protein-derived amino acids into muscle tissue. For instance, prior exercise and neuromuscular electrical stimulation have been shown to result in greater *de novo* muscle protein synthesis[42,43,56,57]. Resistance-type exercise enhances the capacity to incorporate dietary protein-derived amino acids into muscle tissue for at least 12 h[56]. However, post-exercise cooling reduces the incorporation of dietary protein-derived amino acids following post-exercise meal intake[52]. Lastly, short-term muscle disuse results in lower *de novo* muscle protein synthesis[58] (Fig. 4). Therefore, it is evident that physical (in)activity is an important factor modulating the capacity to utilise

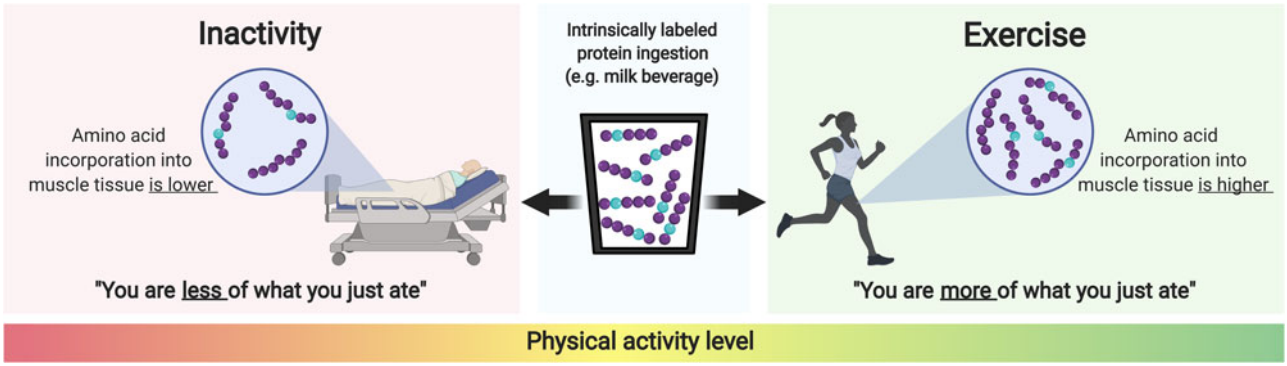

Fig. 4. Schematic representation of the impact of physical (in)activity on the incorporation of dietary protein-derived amino acids into skeletal muscle protein.

dietary protein-derived amino acids as precursors for *de novo* muscle protein synthesis.

Simultaneous assessment of multiple aspects of post-prandial protein handling

A benefit of using a comprehensive oral-intravenous tracer approach is that various aspects of post-prandial protein handling can be assessed within the same experiment under identical experimental conditions. It is evident that protein digestion and amino acid absorption modulate most of the downstream elements of post-prandial protein handling. Dietary protein-derived amino acid release into the circulation appears to be the main determinant of whole-body protein balance and dietary protein-derived amino acid incorporation into muscle tissue. All three have demonstrated a dose–response relationship with no sign of an upper limit with doses ingested up to 45 g dietary protein[6,25–27]. In contrast, maximal stimulation of fractional muscle protein synthesis rates seems to occur at an ingested dose of about 20–30 g dietary protein[6,25,59,60]. Ingestion of greater dosages of intrinsically labelled protein further increases dietary protein-derived amino acid incorporation into muscle tissue protein without a concomitant increase muscle protein synthesis rate[21,27,51]. This is the direct result of a greater post-prandial release of labelled amino acids and, as such, greater post-prandial precursor availability derived from the ingested protein source. Although resistance-type exercise is a strong anabolic stimulus that increases muscle protein synthesis rates and the incorporation of dietary protein-derived amino acids into muscle tissue, it does not result in a detectable increase in whole-body protein synthesis or net balance[42,43]. Taken together, these inconsistencies between various aspects of post-prandial protein handling teach us that we should be cautious when attempting to extrapolate between different metabolic outcomes.

Conclusions

Post-prandial protein handling *in vivo* in human subjects can be assessed by combining stable isotope-labelled amino acid infusions with the ingestion of intrinsically labelled protein with frequent collection of blood and muscle tissue samples. The combined ingestion of intrinsically labelled protein with intravenous stable isotope amino acid infusions allows for the simultaneous assessment of protein digestion and amino acid absorption kinetics (total amino acid rate of appearance, exogenous protein-derived amino acid rate of appearance, endogenous amino acid rate of appearance and total amino acid rate of disappearance), whole-body protein metabolism (whole-body protein synthesis, breakdown and oxidation rates and net protein balance) and skeletal muscle metabolism (muscle protein fractional synthesis rates and dietary protein-derived amino acid incorporation into muscle protein). Matching the labelled amino acid in intrinsically labelled protein with the intravenously infused labelled amino acid allows for a better maintenance of tracer steady-state conditions of the precursor pool and allows for a more accurate quantification of post-prandial muscle protein synthesis rates. The application of intrinsically labelled protein and intravenous stable isotope-labelled amino acid infusions has revealed that dietary protein-derived plasma amino acid availability can be strongly modulated by numerous nutritional and non-nutritional factors. This is of important clinical relevance, since dietary protein-derived amino acid availability is the main determinant driving the post-prandial increase in whole-body and skeletal muscle protein synthesis rates. The combination of ingesting intrinsically labelled protein with the continuous infusion of labelled amino acids is now frequently being applied to investigate how muscle protein synthesis rates are modulated by the various aspects of post-prandial protein handling and metabolism. It is therefore evident that the use of multiple stable isotope amino acid techniques, including intrinsically labelled proteins, will be critical in our efforts to understand various nutritional and non-nutritional factors impact post-prandial protein handling.

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Conflict of Interest

None.

Authorship

J. T., A. M. H., P. J. M. P. 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 work. Figures were created with Biorender.com.

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