Measuring muscle protein synthesis in humans and the influence of nutritional state

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LIST OF ABBREVIATIONS
DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; KIC, α-ketoisocaproate; mTOR, mammalian target of rapamycin

ABSTRACT

In 1982 and 2011 Clinical Science published highly cited papers that used infusion of stable isotope-labeled amino acids to assess skeletal muscle protein synthesis in the fasted and fed state and before and after a period of increased intake of omega-3 fatty acids, respectively. The earlier paper introduced stable isotope tracer approaches in humans that showed consuming a meal will increase whole body oxidation, synthesis and breakdown of protein, but that protein synthesis is greater than breakdown resulting in net accumulation of protein. The paper also demonstrated that consuming a meal promotes net protein synthesis in skeletal muscle. The later paper introduced the concept that omega-3 polyunsaturated fatty acids are able to improve anabolism by reporting that 8 weeks consumption of high dose omega-3 fatty acids by healthy young and middle aged adults increased skeletal muscle protein synthesis during a hyperaminoacidemic-hyperinsulinemic clamp compared to what was seen during the clamp at study entry. Omega-3 fatty acids also increased the phosphorylation of important signaling proteins in muscle, including mammalian target of rapamycin, p70s6k and Akt, during the clamp. These two papers remain relevant because they offer experimental approaches to study human (patho)physiology in different contexts, they present novel insights into the impact of nutritional state (feeding) and specific nutrients (omega-3 fatty acids) on muscle protein synthesis, and they suggest ways to explore the potential of interventions to help prevent and reverse the age-, disease- and disuse-associated decline in muscle mass.

MEASURING MUSCLE PROTEIN SYNTHESIS IN HUMANS AND THE INFLUENCE OF NUTRITIONAL STATE

In 1982, *Clinical Science* published a paper by Rennie et al. (1) describing the measurement of whole body and skeletal muscle protein synthesis in human adults using an infusion of ^{13}C-labeled leucine and how protein synthesis differed in the fed and fasted states. The paper is cited extensively because of the introduction of stable isotope tracer approaches in humans to measure metabolism in different conditions. The authors applied these stable isotope tracer approaches in response to an anabolic intervention and they chose the most obvious one, a meal. A Web of Science search on 2 June 2022, identified 410 citations of this paper, marking it out as the 3rd most highly cited paper published in *Clinical Science* in the 1980s and the 12th most highly cited paper of all time from the journal. The pattern of citations indicates that this paper has an enduring impact (**Figure 1**). In 2011, *Clinical Science* published a paper showing that muscle protein synthesis, also measured with a stable isotope, in response to an anabolic stimulus is increased by supplemental omega-3 polyunsaturated fatty acids (2), a concept that was not proven in humans at the time of the publication. Web of Science identifies 208 citations of this paper, making it the 13th most highly cited paper published in Clinical Science in the period of 2010-2019. This paper cited the paper by Rennie et al. with regard to the technical approach used to study skeletal muscle protein synthesis in humans.

Rennie et al (1) was a collaboration between groups in the UK and USA and included several of the major players in the field at that time. The purpose of the research was to underline the importance of muscle tissue in the response to a meal. Although results of animal research were available at the time, the research group wanted to test whether getting information about the response of human muscle tissue to a meal ~~in human muscle tissue~~ was feasible. They therefore applied a method, using a non-radioactive stable tracer of the essential amino acid leucine (L-[1-^{13}C]Leucine) and blood and breath sampling.

When studying metabolism, either the tracer or the tracee (the meal) should be given in a steady-state fashion. The authors chose to continuously infuse the stable tracer and to provide hourly sips of a liquid, commercially available, oral nutritional supplement. Thus, both tracer and tracee were in steady state. The total duration of the study was about 8 hours and the study was performed in the same subjects either fasted or fed, using a cross-over design. In the fed study, the total amount of protein given was about 45 g, which represents about 0.5 g protein/kg body weight for the 8 hour of the study.

During a study day, plasma samples were collected and the enrichment of leucine and of α-ketoisocaproate (KIC) was measured. The enrichment of KIC (the product of leucine transamination in muscle) was used to establish the muscle precursor enrichment as the intracellular muscle enrichment of leucine (the true precursor of the measured protein synthesis) appeared to be in the same range as the plasma KIC enrichment.

The whole body flux (in the fasted condition this is from protein breakdown and in the fed condition from the meal and protein breakdown) increased after the meal (**Figure 2**). Further information from this figure, which uses data from Rennie et al (1) highlights some important concepts. Consuming a meal will increase the oxidation, synthesis and breakdown of protein. However, because the increase in protein synthesis is larger than the increase in protein breakdown, protein balance becomes positive after a meal (i.e. the meal has an anabolic effect). The increase in the net balance from fasting to feeding (from -14 to +15 μmol/kg body weight) is about 46% of the increase of protein synthesis.

Protein synthesis in muscle also showed an increase that was in the same order as the increase of whole body synthesis (**Figure 3**), suggesting that there is an anabolic effect in skeletal muscle, as in the whole body. Interestingly, the confidence intervals of the measurements made in muscle are somewhat larger than those of the whole body measurements, suggesting that the variability of measuring protein synthesis in muscle is larger and thus it may be somewhat more difficult to observe differences in muscle protein synthesis after a meal. It could be argued that whole body measurements after a meal can better estimate the anabolic response to a meal than muscle protein synthesis measurements.

Rennie et al (1) were able to calculate the actual increase in the net protein synthesis in grams in muscle and other tissues (**Figure 4**). As the subjects received about 45 g of protein, the calculations indicated that the increase in whole body protein synthesis in the fed state is almost double the amount of protein consumed and that muscle is the major contributor to whole body protein synthesis with about a 50% increase in protein synthetic rate for both the whole body and skeletal muscle.

The data of Rennie et al (1) clearly support the notion that protein consumed in food can lead to net protein synthesis in both skeletal muscle and the whole body, to which skeletal muscle is a major contributor. This important finding, which is fundamental to the understanding of protein metabolism in different physiological states (feeding, fasting, exercise) and in illness most likely explains why this paper continuously receives citations, even after 40 years (**Figure 1**).

Many research studies (too many to cite) have followed on the same topic as Rennie et al (1) and use the same, or a similar, approach, although not all of these papers cite Rennie et al (1). The studies often have modifications of the protocol described by Rennie et al (1) that need to be briefly discussed (**Table**).

Giving food as an hourly sip feeding as done by Rennie et al (1) has been replaced in some newer protocols by sip feeding more frequently (e.g. every 20-30 min) in order to better achieve a steady state. Also as sip feeding is not viewed as a physiological way of providing food (in contrast to providing continuous enteral feeding as in some clinical settings), studies have been done after a bolus meal, rather than with continuous sip feeding. However, a clear benefit of using a bolus meal to replace the sip meal approach has not been established (3). In addition, stable isotope tracers are now often added to the meal to be able to calculate additional parameters of the response to the meal such as endo- and exogenous amino acid flux and splanchnic extraction (3). Some studies have indicated that adding free amino acids to a bolus meal is problematic and that the oral stable tracer should be incorporated into the meal protein (e.g. milk, egg or meat proteins) (4). However, it remains unclear whether this is beneficial for tracer studies and therefore whether the expensive procedure to include stable tracers in food protein is worth the effort. Other limitations with this include that there is no steady state achieved for the oral tracer (either as free amino acid or within the digested protein), that the apparent splanchnic extraction of the oral tracer could simply reflect an exchange of the tracer in the splanchnic area (5), and that the enrichments of the given stable isotope tracers need to be measured for a period of time to include the full uptake of the meal (3). Also there can be a difference in the protein digestion rate in certain disease states (6), which seems not to be taken into account in many studies. Therefore, despite modifications by others, the methodological approach described by Rennie et al (1) remains valid in our view; net protein synthesis after a meal can be measured by only using intravenous infused stable isotope tracers.

Many studies have used the combination of a leucine tracer and breath collection and analysis. This approach is robust, but has the disadvantage of breath collection, which, although non-invasive, could be difficult in some clinical settings. Therefore, many studies also use intravenous infusion of the combination of stable tracers of phenylalanine and tyrosine (see for example Smith et al (2)). This method requires blood sampling, which although invasive, is well suited in most clinical settings and patient groups. The results of using leucine or phenylalanine stable tracers approach seems to be comparable (7, 8).

The measurement of muscle protein synthesis can be done with either a leucine or a phenylalanine tracer. A critical issue is that when a meal is given as a bolus, there is no steady state of tracee in the muscle tissue as the tracee from the food disturbs the steady state of the tracer. For

this reason the approach of Rennie et al (1) to provide the food as regular sip feeds makes sense. It still remains unclear whether providing the meal as a bolus in contrast to giving the meal with sips will provide more insight into the skeletal muscle response to food.

One interesting finding of Rennie et al (1) is that the increase of protein synthesis in muscle is larger than the amount of protein provided by the meal, suggesting that the increase in muscle protein synthesis includes a contribution separate from the protein meal. It could well be that after the meal, there is also an increase in the protein breakdown rate in muscle, as was observed at the whole body level (**Figure 2**). Although many papers use an increase in muscle protein synthesis as a direct proof of the anabolic effect of the studied meal, the lack of information about protein breakdown makes it difficult to draw a firm conclusion about net anabolism.

In conclusion, Rennie et al (1) published in 1982, still sets the stage in this area of research, indicating the importance of that work.

Almost 30 years after Rennie et al (1), Smith et al (2) reported on the effect of chronic supplementation with omega-3 fatty acids on the muscle protein anabolic response to amino acids and insulin. The bioactive omega-3 fatty acids are eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). EPA and DHA are found in fatty fish, in omega-3 supplements ("fish oils") and in concentrated pharmaceutical preparations used clinically for triglyceride lowering. They have many physiological impacts that mainly relate to their appearance in cell membranes subsequently influencing cell signaling, gene expression, lipid mediator synthesis and cellular responsiveness (9). EPA and DHA could affect muscle protein synthesis through influencing whole body or muscle physiology (e.g. improving insulin sensitivity) or through direct impacts on the machinery of protein synthesis (10). Increased intake of EPA and DHA from supplements over a period of several weeks increases the amounts of those fatty acids in skeletal muscle (2, 11, 12), presumably in membranes. This has been shown to increase the levels of some signaling proteins including focal adhesion kinase and mammalian target of rapamycin (mTOR) (12).

In this field, Smith et al (2) is an important piece of research. Prior to this human research, studies in laboratory rodents had reported that EPA and DHA helped to maintain whole body protein synthesis, whole body protein balance and muscle mass under conditions of catabolic stress (13). In a separate human study Smith et al. (11) had reported that daily supplementation of high dose EPA+DHA (3.4 g/day for 8 weeks to healthy older adults (age > 65 years) increased the rate of muscle protein synthesis that occurred in response to a hyperaminoacidemic-hyperinsulinemic clamp. The increase was about 240% above the rate observed with the clamp at study entry. There was no effect of omega-3 fatty acids on the rate of protein synthesis observed in the absence of the clamp. In

this study, EPA and DHA increased the serine 2448 phosphorylation of mTOR and the threonine 369 phosphorylation of p70s6k seen in response to the clamp (11).

The study reported in Clinical Science (2) used a very similar design but was conducted in healthy younger adults (25 to 45 years old). Again, the participants received 3.4 g EPA+DHA per day for 8 weeks and the muscle protein synthesis rate was studied using infusion of stable isotope labeled phenylalanine prior to and then during a hyperaminoacidemic-hyperinsulinemic clamp. At study entry, the clamp increased muscle protein synthesis rate by about 70%. After the 8 weeks of omega-3 fatty acid intake, protein synthesis during the clamp was increased by about 100% and this increase was about 45% higher than at study baseline (**Figure 5**). The clamp increased serine 2448 phosphorylation of mTOR, threonine 369 phosphorylation of p70s6k and threonine 208 phosphorylation of Akt. However, the phosphorylation of these signaling proteins in response to the clamp was greater after the period of omega-3 fatty acid intake compared to study entry. Thus, Smith et al (2) provide evidence that bioactive omega-3 fatty acids significantly increase the muscle protein anabolic response to a hyperaminoacidemic-hyperinsulinemic clamp in young and middle aged adults, having already demonstrated this in older adults (11). Further, they link this metabolic effect to altered activation of proteins involved in the signaling linking amino acids and insulin to the pathway of protein synthesis. It is important to note that omega-3 fatty acids did not affect the basal rate of protein synthesis, so their action seems to be linked to augmenting the anabolic effect of other signals. Smith et al (2) also used labeled glucose and found no effect of omega-3 fatty acids on whole body glucose disposal, seeming to rule out an effect of EPA and DHA on insulin sensitivity. Smith et al. (2) did not measure muscle mass in their study, perhaps because the number of participants was quite low (n = 9).

In a more recent study, McGlory et al. (14) reported that intervention with EPA+DHA (approx. 5 g/day) resulted in better maintenance of quadriceps volume during 2 weeks of leg immobilization and that this was linked to retention of a higher rate of myofibrillar protein synthesis during the immobilization period. This observation suggested that the effect of omega-3 fatty acids on muscle protein synthesis could be relevant to situations where there is loss of muscle mass due to aging, disuse or disease (14). In this context, a recent meta-analysis of human studies in heterogeneous population groups identified that lean mass and skeletal muscle mass are favored by higher intakes of EPA and DHA (15).

One limitation of the study of Smth et al. (2) is the small sample size. However these types of studies are technically challenging and not inexpensive, and, despite its sample size, the key findings were statistically significant. A second limitation is that the dose of omega-3 fatty acids used by Smith et al. in both their studies (11) (2) is high compared with doses that can be readily consumed by most individuals. Habitual intakes of EPA and DHA in most populations are considered to be < 200 mg per

day although they will be higher in regular consumers of oily fish or of omega-3 supplements. Nevertheless it would be very difficult for most people to achieve an intake of 3.4 g EPA + DHA per day, although pharmaceutical preparations of omega-3 fatty acids and some enteral feeds might allow this. More literature is emerging on a role for omega-3 fatty acids in supporting muscle protein synthesis and muscle maintenance in aging and in different clinical settings that adversely impact muscle mass and function (10, 15); currently omega-3 fatty acids are not specifically included in recommendations for these groups because of their effects on protein synthesis and on muscle and protein synthesis. However, as more research supporting such a role for omega-3 fatty acids emerges, such recommendations might come.

In summary the publications of Rennie et al. (1) and Smith et al (2) remain relevant because they offer experimental approaches to study human (patho)physiology in different contexts, they present novel insights into the impact of nutritional state (feeding) and specific nutrients (EPA and DHA) on muscle protein synthesis, and they suggest ways to explore the potential of interventions to help prevent and reverse the age-, disease- and disuse-associated decline in muscle mass. For these reasons, it seems likely that both these papers will continue to be cited for years to come.

Table: Modifications to the stable isotope protocol of Rennie et al. (1) and possible benefits			
Original approach	Modified approach	Why was it modified?	Beneficial?
Sip feeding frequency hourly	Sip feeding frequency every 20-30 min	To better achieve a steady state in the intake of nutrients	Yes
Sip feeding of the meal	Bolus intake of the meal	A more natural meal consumption	Unclear
Only intravenous stable isotope tracers used	Add stable isotope amino acid tracers to the meal	To compensate for splanchnic extraction of meal derived amino acids	Remains unclear whether this approach actually measured the extraction of meal-derived tracee amino acids
	Add the stable isotopes amino acid tracers as part of protein in the meal	To have a comparable absorption of tracer and tracee amino acids	Only when the absorption pattern is very different (e.g. casein vs whey protein)
Leucine stable isotope tracers + breath analysis	Phenylalanine + Tyrosine stable isotope tracers	To be able to obtain information only from blood analysis	Yes
Muscle protein synthesis measured after sip feeding	Muscle protein synthesis measured after a bolus meal	A more natural meal consumption	When lacking a steady state of isotopes in the muscle precursor pool, the muscle protein synthesis measurements may not be accurate
Modifications to the protocol used by Rennie (1)			

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