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Pediatric protein metabolism techniques – a review

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Abstract. – Some invasive techniques could be safely and ethically applied to estimate protein metabolism in children. Although these methodologies provide valuable information on whole body protein metabolism, a reflection of what is happening to thousands of protein molecules across a variety of different tissues. However, they provide little information regarding the contribution of specific tissues and proteins to the whole-body changes observed (e.g., whether the increase in protein synthesis was a result of the muscle or splanchnic tissues) which might otherwise be determined in more invasive procedures such as those with muscle biopsies. Considering ethical constraints in pediatric research, it is not possible to perform stable isotope methods with concurrent muscle biopsies. The present review article enlightens the techniques in use for measurement of pediatric protein metabolism.

Key Words:
Pediatrics, Protein metabolism, Techniques.

Introduction

Classically, nitrogen balance method has been viewed as theoretically the most suitable tool to determine protein requirements[1]. As previously mentioned, the current dietary reference intakes (DRIs) for protein intake in healthy individuals are based on a careful review of the recent meta-analysis[2] of nitrogen balance (NBAL) studies, as this is the only assessment technique to date. Moreover, it has produced enough data to make reasonable estimates of total protein (N) requirements. One major benefits of the NBAL technique are its non-invasive nature and its ability to be administered under free-living conditions.

NBAL is calculated as the difference between N intake (NIN) and N losses (NEX). As the only N entering the body comes from dietary protein, NIN could be easily estimated by assessing the total intake of dietary protein, assuming 16% of which is made up of N[3]. NEX, on the other hand, is calculated as the amount of N excreted in urine, feces, skin and other miscellaneous sources (i.e., sweat, hair, nails, saliva and breath). Urea serves as the major end product for the degradation and disposal of amino acid N and is the dominant form (> 80%) of urinary NEX. Thus, the amount of urinary urea excreted from the body is an indicator of the amount of N being excreted from the body and, as such, an indicator of protein metabolism.

As NEX is primarily (> 84%) a result of urinary metabolites (i.e., urea, creatinine, and ammonia)[4], the concentration of urinary metabolites are usually measured directly using specific assay methods (e.g., spectrophotometry). A small amount of N-containing metabolites could also be excreted in the feces and through the integumentary system. Therefore, to acquire a comprehensive indication of N excretion these routes of egress must also be considered. Since fecal and miscellaneous sources of NEX are often small compared with urinary metabolites, these measures were estimated according to previously published values[5], rather than measured directly. Therefore, estimates of sweat NEX using the change in body mass as a result of an exercise in combination with the average sweat nitrogen concentration.

Despite the widespread use of the NBAL methodology, there are various practical limitations and problems to the determination of NBAL. The first limitation is that in order to attain a proper steady state for NEX to allow for sufficient turnover of urea, individuals should be adapted for several days to each new level of dietary protein intake being investigated[6]. Secondly, NBAL often overestimates NIN and underestimates NEX resulting in a consistent overestimation of NBAL reported in the literature (2). A third limitation of this technique is that an individual should be studied at various levels of protein consumption (i.e., spanning deficiency to excess) in order to interpolate estimates of individual requirements[7]. Finally, the

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NBAL technique has been criticized with regards to the linear modeling used to estimate protein requirements, due to the decreased efficiency of protein utilization approaching zero balance[8]. Notwithstanding these limitations, NBAL is still used today as an indicator of an individual's nutritional state and to establish requirements for protein.

Whole Body Protein Turnover: the Use of Stable Isotopes

Stable isotopes are heavier forms of a given element containing one or more neutrons in their nucleus. Most elements, both in nature (e.g.. foodstuffs) and in the human body (e.g., tissues), already contain a mixture of various stable isotopes at low, yet significant, natural levels[9]. For application in the study of physiology and metabolism stable isotopes are commonly used in the form of a tracer. Tracers are produced by chemically attaching the isotope to a compound of interest. This is followed by administration of the tracer by either ingestion or infusion[10]. As the mass difference between the stable isotope and predominant natural form is often small for common isotopes (e.g. ^{15}N *vs.* ^{14}N and ^{13}C *vs.* ^{12}C), the physicochemical properties of the tracer are nearly identical to the unlabeled tracer under investigation[11]. The assumption, therefore, is that both the tracer and unlabeled tracer would act in a similar manner with respect to metabolism[12]. By measuring the amount of tracer and their subsequent metabolites, estimates of the utilization of the compound of interest could be determined[13].

As proteins and their associated amino acids are constantly undergoing turnover, static measures provide little information on the dynamic processes taking place[9]. Tracers, on the other hand, provide more detailed information regarding the metabolic fate of the particular nutrient of interest (e.g., N or specific amino acids). Moreover, in contrast to radioisotopes, these isotopes are not radioactive when they disintegrate. So, these could be used in infants and children as well as other special populations (i.e., pregnant women)[11]. Further, [^{15}N] glycine and L-[1-13C] leucine are the most commonly used stable isotopes tracers to assess protein metabolism[14], especially in pediatric research.

Oral [^{15}N] Glycine Tracer

The 'end-product' approach with [^{15}N] glycine is a commonly used method to measure components of whole body protein turnover including whole body N flux (Q), protein synthesis, protein breakdown and subsequently WBPB. The end-product method is based on the assumption that the proportions of the tracer dose that go to synthesis and excretion are identical to the proportions of flux that go to synthesis and excretion[15]. One important thing to note when using the end-product method is, the time of the collection, as the time course of the excretion of ^{15}N in urinary ammonia and urea is not uniform[15]. Ammonia undergoes a rapid turnover, and by 9-12 h the excretion of [^{15}N] ammonia is essentially complete. When measurements exceed this point, a small but considerable error could be introduced due to the breakdown of labeled protein and, thus, recycling of the tracer into the ammonia pool.

Urea, on the other hand, undergoes a slower turnover whereby about 25% of [^{15}N] urea from the administration of the tracer is still retained in the body after 9-12 h Therefore, in during the collection between 9-24 h, the risk of tracer recycling in the urea pool is negligible. The difference in time course of excretion between N-ammonia and N-urea is based on the notion that rather than a single homogeneous 'metabolic' pool, there are instead two pools that are (to some extent) spatially and metabolically separate. It has been further proposed that QA represents the metabolism of the peripheral tissues (i.e., muscle), whereas QU better represents the splanchnic metabolism.

As the estimates of Q based on ammonia (QA) and urea (QU) vary, there are two different approaches that could be used. The decision on which approach to use often depends on the time frame of interest. The first one is the ^{15}N-ammonia end-product method[16], which only uses the value for ammonia-N in the calculation of NEX. The other approach that is often used measures both urea-N and ammonia-N, and estimates total N-excretion using an average (either arithmetic or harmonic) of the two to best represent a 'true' Q. This approach is generally used to assess whole body protein metabolism over a longer time of 16 h or more.

One of the major benefits of the [^{15}N] glycine end-product approach, particularly for use in children, is its non-invasive nature, whereby all metabolic endpoints can be collected and measured using urine samples[15]. Moreover, participants are unrestricted in their movements throughout the procedure (aside from when urine samples are collected) allowing them to perform collections outside of the laboratory. Finally, it has the ability for protein kinetics to be measured over relatively long time frames (e.g., up to 24 h)[16].

Indeed, there are some limitations that must be considered when using the [^{15}N] glycine methodology[14]. One setback of the end-product approach is the practical difficulties in collecting samples outside of the laboratory and the assumption that the sample has been collected in its entirety. For this reason, it is often easier for the assessment to occur overnight that, for children[10]. Another limitation to this technique is with respect to the sensitivity of the oral [^{15}N] glycine tracer in its ability to detect small differences in protein turnover that might otherwise be detected by methodologies such as IV infusions. Finally, the [^{15}N] glycine technique generally has a low time resolution (due to the necessity to adequately collect the metabolic end-products) over which changes in protein turnover could be measured. Despite its limitations, it has been argued that if the [^{15}N] glycine technique is applied in a strict and standardized way, reasonable estimates of whole-body protein turnover could be determined.

Intravenous L-[1-13C] Leucine Infusion

Currently, the 'gold standard' for assessing whole-body protein kinetics and quantifying rates of protein turnover in humans is the 'precursor' method. Leucine is typically used as the labeled amino acid as it is an essential amino acid and, therefore, could not be produced *de novo* by the human body. Secondly, it has a relatively greater rate of oxidation and it is preferentially oxidized within the skeletal muscle[17]. This method could provide reliable estimates of leucine oxidation (LeuOX), estimates of leucine balance (LeuBAL) and flux at the whole-body level by using only short-term measurements during either at the fed or post-absorptive periods.

The use of IV infusion of [1-13C] leucine to assess whole-body protein kinetics relies on several assumptions. The majority of these assumptions are generally accepted and considered valid in most physiological conditions even after years of intense scrutiny. Due to the collection of blood samples, one advantage of this technique is the ability to measure changes in circulating amino acid and insulin concentrations that are not available when using the end-product method. Another advantage of this technique is a better time resolution than the [^{15}N] glycine methodology. In conjunction with non-steady-state kinetics, this technique could assess changes in LeuOX, endogenous rate of appearance (Endo *Ra*; marker of protein breakdown) and non-oxidative leucine disposal (NOLD; marker of protein synthesis) over 20-30 min intervals[18]. This makes it ideally suited to determine the acute effects of a single nutrition intervention on protein metabolism.

Although the majority of amino acid degradation occurs in the liver[19], amino acid degradation also occurs in other tissues. For example, a special subset of essential amino acids known as BCAAs (i.e., leucine, isoleucine, and valine) are unique in a small fraction of their catabolic capacity but the majority occurs in extra-hepatic tissues[20]. Skeletal muscle is a major site of BCAA catabolism as approximately half of BCAA catabolic capacity resides in this tissue. The BCAT enzyme catalyzes the reversible transamination of BCAAs, whereas BCKD is the rate-limiting enzyme for BCAA oxidation located in the mitochondrial membrane that catalyzes a decarboxylation of each of the three α-ketoacids and commits them to oxidation[21].

Despite the academic advantages, however, there are some practical disadvantages of the technique. Compared with the end-product method, the precursor method is more invasive in nature. Though this technique is still considered to be minimally invasive, it does pose the risk of physiological discomfort and potential emotional distress to the child[10]. Furthermore, a collection of breath samples is also needed for the [1-13C] leucine method. Although this might be easy to perform and convenient for healthy subjects, it becomes more complex in patients who are ill, weak ventilated or anesthetized[14]. Finally, complex and relatively expensive equipment is needed to perform the technique[15]. As participants have restricted movement during the time in the laboratory, it also places limitations on the exercise procedures that could be used. The [1-13C] leucine method requires the use of three analytical techniques. Indirect calorimetry is used to determine total CO_2 production; an isotope-ratio mass spectrometer (IRMS) is utilized to assess the isotope enrichment in expired CO_2; and a gas chromatograph-mass spectrometer (GCMS) is exploited to measure the precursor pool plasma KIC.

Conclusions

Fortunately, major methodological advancements of these analytical methods (particularly IRMS and GCMS) allows the detection of labeled isotope tracers and their metabolites with highly accurate measurements using very small amounts of sample material. This makes their use in pediatrics a particular benefit, as the very little biological sample is required to be collected.

Conflict of interest

The authors declare no conflicts of interest.

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