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Chapter 2

Proteome Dynamics with Heavy Water — Instrumentations, Data Analysis, and Biological Applications

T. Kasumov, B. Willard, L. Li, R.G. Sadygov and S. Previs

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http://dx.doi.org/10.5772/61776

Abstract

The quantitative assessment of the synthesis of individual proteins has been greatly hindered by the lack of a high-throughput nonradioactive method. We recently developed a method that we call "proteome dynamics" and software that enables high-throughput kinetic analyses of peptides on a proteome-wide scale. Previous studies established that oral administration of heavy water (^2H_2O or deuterium oxide, D_2O) is safe and well tolerated in humans. Briefly, a loading dose of ^2H_2O, a nonradioactive isotope, is administered in drinking water. ^2H_2O rapidly labels body water and transfers ^2H from ^2H_2O to ^2H-labeled amino acids, which incorporates into proteins dependent upon the rate of synthesis of the specific protein. Proteins are analyzed by high-resolution mass spectrometry and protein synthesis is calculated using specialized software. We have established the effectiveness of this method for plasma and mitochondrial proteins. We demonstrated that fasting has a differential effect on the synthesis rates of proteins. We also applied this method to assess the effect of heart failure on the stability of mitochondrial proteins. In this review, we describe the study design, instrumentation, data analysis, and biological application of heavy water-based proteome turnover studies. We summarize this chapter with the challenges in the field and future directions.

Keywords: Heavy water, proteome dynamics, protein synthesis, modeling, isotopomers, mass spectrometry

1. Introduction

Prior to isotope studies, it was believed that the protein pool in the body was in a static state without any dynamic changes [1–3]. The pioneering work of Schoenheimer and his colleagues investigated the metabolic activities of body proteins using amino acid tracers and therein

established the dynamic nature of the protein pool [4, 5]. Subsequent experiments thoroughly studied the protein balance in the body and revealed that the diet only provides 60–80 g of proteins (per day) as a source of amino acids building blocks for protein synthesis, while the human body synthesizes 300–500 g of protein every day [6]. This discrepancy between dietary protein supply and synthesis suggests that the majority of the newly made proteins are synthesized from amino acids which are derived from degradation of preexisting proteins [7]. In addition, the *de novo* synthesis of nonessential amino acids from ammonia and intermediary metabolites derived from the glycolytic pathway, the pentose pathway, and the citric acid cycle also contributes to protein synthesis [8] (Fig. 1). It is now well recognized that protein turnover —synthesis and degradation—is critical for the maintenance of all cellular processes [9].

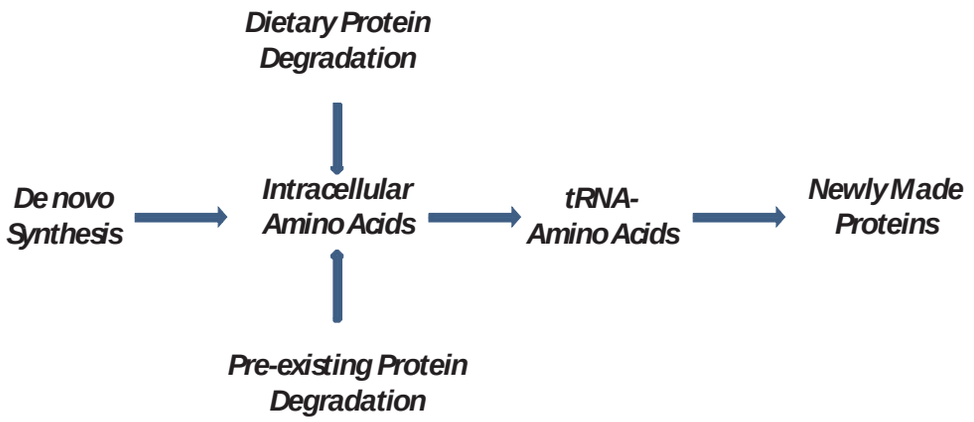

Figure 1. Sources of intracellular amino acids for protein synthesis.

The total protein synthesis rates in whole body and different organs have been measured using radioactive (^{14}C, ^{35}S, and ^{3}H) and stable (^{13}C, ^{2}H, and ^{15}N) isotope labeled amino acids in a tissue using the labeling ratio between the precursor amino acids and the protein products [10]. Because of the simplicity, radioactive isotopes dominated early protein turnover studies until gas chromatography-mass spectrometry (GC-MS) became commonly available for stable isotope-based tracer studies [11]. Radioactive amino acids were widely used in pulse-chase experiments that enabled quantification of both protein synthesis and degradation. However, due to safety concerns, radioactive isotopes found limited application in human studies. With the advancement in mass spectrometry instrumentation, the stable isotope-based amino acids found widespread use in clinical research. Similar to radioactive isotopes, two major designs, i.e., flooding dose or primed infusion of the stable isotope labeled amino acids are utilized to study protein turnover in human studies. Multiple studies investigated advantages and disadvantages of both methods [12, 13]. With a different degree of success, both methods enhanced our understanding of total protein dynamics in different tissues and circulation. However, both methods have been associated with several problems related to the assessment of true precursor enrichment and its impact on data interpretation; in addition, experimental design typically requires inpatient tracer administration. As discussed below, this is particularly critical for the short-term labeling protocol that is based on a precursor and product

http://dx.doi.org/10.5772/61776

relationship. The "true precursor" for protein synthesis is the intracellular tRNA-bound amino acids which are usually not accessible, particularly in human studies. Therefore, several extracellular surrogate markers of the "true precursor" have been used for calculation of the kinetic parameters with varying success. Finally, these methods generally require a large amount of expensive tracers, and in the case of stable isotopes, infusion of labeled amino acids elevates amino acid levels and perturbs normal protein metabolism. Until recently, all of these methods were only applicable in studies of total protein kinetics (i.e., consisting of a mixture of proteins) without giving any knowledge about the turnover rates of individual proteins. This shortcoming has particular relevance to health and disease, since it is recognized that proteins differentially respond to stress and the averaging of individual protein fluxes may result in a cancellation of changes in their kinetics. This point can be easily illustrated in the case of acute-phase response (APR) proteins. Due to the distinct dynamics of positive and negative APRs, they are differently affected in conditions associated with inflammation [14] or fasting [15]. Although advancement in methods surrounding protein isolation and sample preparation allowed the analysis of purified (individual) proteins, these methods are in general cumbersome, labor-intensive and, in many cases, it is difficult to purify proteins (specifically low abundant ones) from other contaminants.

Over the last 25 years, the development of novel analytical proteomics methods has provided a major advancement in medical research by allowing investigators to quickly identify and measure the relative amount of a large number of proteins in a plasma or tissue sample. On the other hand, like Western blots, these methods only provide static data on protein levels, and no information on the temporal changes on a given protein. By contrast, coupling of static proteomics with stable isotope-based metabolic labeling approaches enables the study of temporal protein dynamics on a proteome scale. Stable isotope labeled amino acids in cell culture (SILAC) [16] and ^{15}N-labeled algae feeding [17] were successfully applied to study protein turnover in cell culture and then *in vivo* in rodents. Although these methods enable quantification of virtually all identified proteins, the study of protein dynamics *in vivo* in humans is challenging. Since all amino acids have nitrogen, ^{15}N-labeled algae feeding enables tracing all proteins and label amplification in a newly synthesized peptide results in a mass shift relative to unlabeled peptides that simplifies the data interpretation. While ^{15}N-labeled algae provide a valuable tool for *in vitro* cell and *in vivo* rodent experiments, it is not practical in human studies because this would require the consumption of a fully ^{15}N-labeled diet. Although the SILAC method has been used in *in vivo* studies [18], the dietary administration of the SILAC tracers, e.g., [$^{13}C_6$]-lysine [19], [$^{2}H_8$]-valine [18], [$^{2}H_3$]-leucine [20], or [$^{13}C_6$]-arginine [19, 21], limits their application only to fed state which prevents comparisons of proteome dynamics in fed vs. fasted state [22]. In addition, the dietary tracer administration of ^{15}N-labeled algae and SILAC also prevents the modification of the diet as an experimental variable which limits the application of these methods to metabolic diseases that require the assessment of the role of multiple physiological parameters including glucose, insulin, and ketone body on protein synthesis in fasted state. Finally, the dietary administration of tracers in both methods does not allow to readily achieve a steady-state labeling in the precursor pool, a critical assumption made in protein turnover calculations based on precursor and product relationships. Deviation from a steady-state labeling in the precursor pool results in underes-

timation of protein synthesis using these methods and/or leads to complications in the mathematical modeling that is required to interpret the data.

Among all other tracers, $^{2}H_2O$ and $H_2{}^{18}O$ have been used to study the protein turnover [22, 23]. The ubiquitous presence of H and O atoms in amino acids allowed investigators to consider both $^{2}H_2O$ and $H_2{}^{18}O$ as unique tracers for the synthesis of virtually all proteins [2, 7, 24]. Since ^{18}O (M+2) isotope adds at least 2Da to each amino acid, the utilization of $H_2{}^{18}O$ results in a larger mass shift that improves the sensitivity of the assay as compared to $^{2}H_2O$. However, $H_2{}^{18}O$ is a relatively expensive tracer and is not necessarily affordable for use in humans.

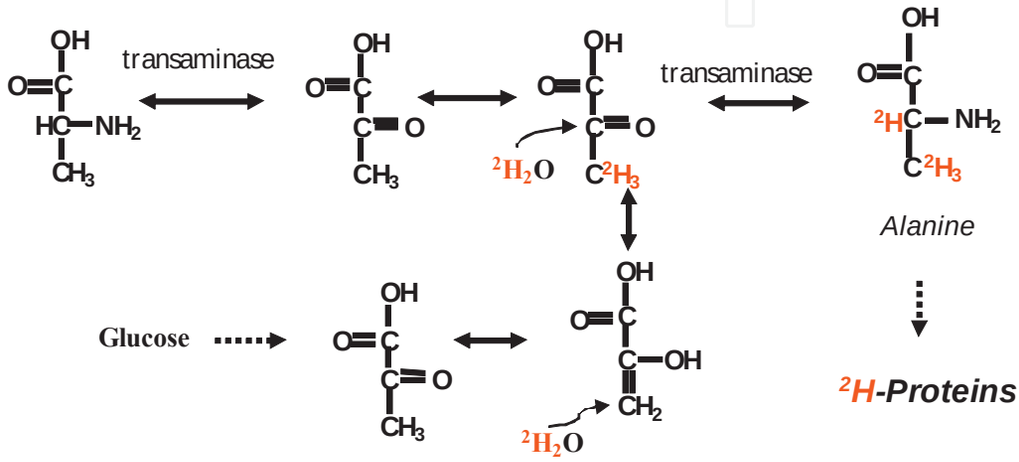

Figure 2. A simplified scheme of ^{2}H-labeling of alanine and proteins.

By contrast, $^{2}H_2O$ is a low-cost tracer which makes it practical for human application [25]. Similar to $H_2{}^{18}O$, $^{2}H_2O$ is safe and it easily equilibrates with total body water (TBW) and $^{2}H_2O$ also rapidly labels all amino acids (e.g., ~10–20 min in rodents and 1 h in humans) [15, 26]. Thus, the quick steady-state labeling of non-exchangeable H atoms in free amino acids after $^{2}H_2O$ administration demonstrates that the rate limiting step of ^{2}H incorporation into proteins is protein synthesis from amino acids (Fig. 2). Although the use of $^{2}H_2O$ in metabolic studies has a long history [24, 27], recently the $^{2}H_2O$-metabolic labeling experienced a renaissance, for assessing DNA synthesis [28], gluconeogenesis [29], and lipid turnover [30, 31]. Previously, the $^{2}H_2O$-metabolic labeling approach has been used by us and others to measure the average synthesis rate of mixed tissue proteins [32–35]. We, and others, recently pioneered $^{2}H_2O$ to study the synthesis rates of individual proteins using advanced mass spectrometry-assisted proteomics *in vivo* [15, 22, 36, 37]. We have continued to refine this approach by combining advanced high-resolution LC-MS (liquid chromatography-mass spectrometry)/MS proteomics with *in vivo* $^{2}H_2O$-metabolic labeling to create a new method called "proteome dynamics," which enables quantification of the rate of synthesis of individual proteins.

By giving $^{2}H_2O$ in the drinking water, one can enrich the precursor amino acid pool with ^{2}H and sustain it indefinitely without affecting the total concentration of precursor amino acids. The rationale is based on the observation that in the presence of $^{2}H_2O$, cells generate ^{2}H-labeled amino acids via transamination and/or *de novo* synthesis (Fig. 2). All amino acids, including

http://dx.doi.org/10.5772/61776

essential amino acids, can exchange at least one H atom as a consequence of a transamination reaction. However, since the equilibrium of ^2H incorporation from total body water into C–H sites of amino acids is not complete, therefore lower values of deuterium incorporation were observed for the essential amino acids [15]. For the nonessential amino acids, the asymptotic number of exchangeable hydrogen atoms varies depending on their structure and their metabolic origin. For example, *de novo* synthesized alanine and glutamine may incorporate up to four and five ^2H atoms, respectively. Although N–H, O–H, and S–H sites of amino acids also spontaneously exchange H with ^2H_2O, these labile hydrogen atoms back-exchange with H_2O during the extensive sample preparation process. We have demonstrated that there is no back-exchange of C-bound ^2H atoms to ^1H from water after proteins have been synthesized and secreted, and therefore only C–H sites contribute to metabolic labeling during protein synthesis [31]. For the same reason, the *in vivo* ^2H_2O-metabolic labeling differs from *in vitro* H/D (^2H) exchange methodology that is widely used for protein structure analysis. In contrast to reversible H/D exchange of labile hydrogen atoms in preexisting proteins, the ^2H_2O-metabolic labeling irreversibly transfers ^2H to the carbon backbone of newly synthesized protein.

The incorporation of multiple copies of ^2H atoms into nonessential amino acids increases tryptic peptides ^2H labeling and improves the assay sensitivity. As a safe, nonradioactive tracer, ^2H_2O can be administered in the drinking water to free living organisms without interfering with their lifestyle routines. These valuable characteristics of ^2H_2O-metabolic labeling make it a unique tracer to study the synthesis rates of all proteins in different species, including humans.

2. The study design for heavy water-based proteome turnover studies

Essentially, all tracer-based protein turnover studies rely on establishing precursor (amino acid) and product (protein) relationships. When using a pre-labeled amino acid, one of the major challenges in protein turnover studies is determination of intracellular true precursor enrichment for the kinetic calculations. The true precursor in protein synthesis is an intracellular tRNA-bound amino acid which is in low quantities, and it is not accessible in extracellular fluids [38]. Therefore, the intracellular labeling of free amino acids has been used as the substitute for true precursor enrichment. Although this can be easily done in animal studies, the invasive tissue analysis is not suitable for human studies. In many experiments, only extracellular amino acids are accessible for the precursor enrichment measurements. Since amino acid movement through the cell membrane is a tightly regulated transporter-mediated process, there is an enrichment and concentration gradient of amino acids across the extracellular and intracellular space. To circumvent this issue, several approaches have been proposed to assess true precursor enrichment. For instance, the labeling of an extracellular α-ketoisocaproate (KIC), a metabolite of leucine, was used as a surrogate of intracellular leucine enrichment [39], while intracellular glycine enrichment was assessed based on urinary hippurate metabolite of glycine [40]. In other studies, intracellular amino acids labeling was assessed based on the analysis of protein-bound amino acid in a fast turnover protein like apoB100 [41]. Several studies have demonstrated that different surrogate precursors result in

substantially different kinetic calculations and therefore defining the true precursor and data interpretation are key issues in protein turnover studies [42–44].

In contrast to amino acids, ^2H_2O freely and rapidly equilibrates with the total body water in all organs and cell compartments and transfers ^2H to intracellular amino acids [15, 36]. This underlying assumption has been validated in multiple studies through analysis of total body water and intracellular amino acids labeling at different time points [15, 26, 45]. For the kinetic calculations, we assume that protein levels do not change during the ^2H_2O-metabolic labeling study period, and that there is steady-state flux of all proteins. We have validated this assumption through quantification of plasma proteins abundance using synthetic stable isotope-labeled peptides [31]. In addition, other investigators have performed a direct comparison of the heavy water method with a primed infusion of [$^2H_3$] leucine [45] and/or a flooding dose of [$^2H_5$]-phenylalanine [46]; these efforts suggest the validity and the reliability of the ^2H_2O-metabolic labeling approach.

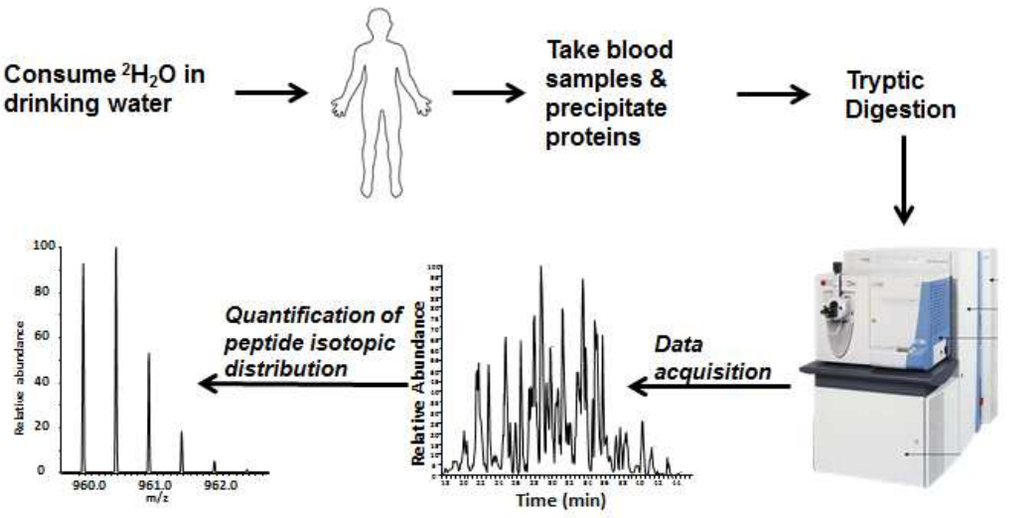

Figure 3. Flow scheme for experimental design and analysis of proteome dynamics with ^2H_2O. After bolus load of ^2H_2O (0.3 ml/kg body weight), human subjects consume 0.5% in drinking water for 1 week and blood samples are collected at different time points.

These experimental results allow investigators to consider ^2H_2O as a precursor of ^2H tracer for proteins synthesis. Recently, we developed an algorithm (details discussed below) for calculating the enrichment of intracellular amino acid based on body water enrichment analysis (from accessible body fluids by simple headspace GC-MS analysis) [37]. This overcomes the issue related to true precursor enrichment. Furthermore, oral administration of heavy water after a bolus load easily maintains a steady-state labeling of total body water and amino acids that result in a substantial enrichment of analyzed proteins. When applied to plasma or serum proteins, the experimental design for ^2H_2O-metabolic labeling is as follows:

- ^2H_2O is given in a bolus dose followed by low intake in the drinking water to maintain a constant steady-state enrichment of ^2H_2O in body water (Fig. 3).

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- Body water and tissue amino acids are rapidly labeled and attain a steady state without perturbing the normal concentration of amino acids. The ^{2}H labeling of free amino acids is directly proportional to the number of carbon-bound exchangeable hydrogen atoms and the $^{2}H_2O$ enrichment of body water, and is independent of the rate of protein synthesis.
- Blood is obtained and proteins are isolated at preselected time points.
- Tryptic peptides are analyzed by LC-MS/MS, which identifies several peptides from each protein of interest, both the endogenous mass and heavier peptides that are enriched with ^{2}H-labeled amino acids. Proteins are identified using online MASCOT or alternative software packages.
- The high-resolution full scan data analysis is performed using in-house-developed software allows quantification of mass isotopomer distribution of peptides.
- The time-course labeling of unique peptides represents the rate of synthesis of a protein that they originate from.

Protein life spans (or half-lives) range from minutes to more than 1 month. Although the heavy water-based metabolic labeling approach may not be suitable for the kinetic studies of very short-lived regulatory proteins such as glucagon, insulin, leptin, and adiponectin, it can capture the kinetics of thousands of proteins with the half-lives that are longer than the distribution and equilibration of $^{2}H_2O$ with amino acids.

This method has major advantages over other stable isotope methods that utilized amino acids pre-labeled with ^{2}H, ^{13}C, or ^{15}N, namely: (1) it enriches all proteogenic amino acids and thus increases the enrichment of newly synthesized proteins to a far greater extent than that can be achieved by infusion or feeding labeled amino acids or proteins, (2) it can be given to humans by multiple oral doses over the course of a day in drinking water and does not require IV infusion, and (3) it is relatively inexpensive (~$350/person) compared to traditional amino acid tracers ($1,000-$4,000/person).

For the most accurate calculation of protein kinetics, two different short-term and long-term experimental designs with heavy water have been employed.

2.1. The short-term heavy water protocol for protein synthesis

The short-term protocol requires the bolus load of heavy water and the measurement of peptide enrichment during the semilinear increase segment of ^{2}H-labeling time-course curve [15, 22]. The optimal design for the short-term heavy water protocol requires multiple time points in the early period of protein synthesis, although a single time-point sampling after $^{2}H_2O$ administration is also possible [47]. For the kinetic calculations, we assume that protein levels do not change during the $^{2}H_2O$-metabolic labeling study period, and that there is a steady-state flux of all proteins. We have validated this assumption through quantification of plasma protein abundance using synthetic stable isotope-labeled peptides. Thus, at a steady state, the rate constant represents both the fractional synthesis rate (FSR) and the fractional catabolic rate (FCR). In this case, the fractional synthesis rate (FSR) of a protein could be

calculated based on the slope of the labeling of the tryptic peptide and precursor amino acid enrichment using the formula [15]:

$$\text{FSR} = \text{slope of } E_{\text{peptide}} / E_{\text{precursor}} \quad (1)$$

where the slope of E_{peptide} is the rate of the increase in ^{2}H-labeling of peptide during ^2H_2O administration and $E_{\text{precursor}}$ is the sum of the enrichment of the amino acids constituting the peptide sequence at the steady state. With this design, collection of multiple samples at early hours of the study enables the estimation of turnover rates of proteins with a short half-life, while extending the experiment for several days or weeks allows the estimation of the kinetics for proteins with slower turnover rates. The FSR also can be calculated based on a single time-point sampling after ^2H_2O administration. However, for accurate estimate of a protein synthesis rate, it is critical to select an appropriate sampling time after ^2H_2O exposure. Since distinct proteins have a wide range of half-lives, this approach may be satisfactory only for selected sets of individual proteins. In addition, sufficient biological and technical replicates are required to achieve good statistics based on one time-point sampling. Although this approach does not require the correction for the baseline enrichment, the net ^{2}H labeling can be calculated via subtraction of the total baseline enrichment before heavy water administration: $E_{\text{peptide}}(t) - E_{\text{baseline}}$. Thus, this approach is very simple and straightforward if sampling points are accurately selected based on the half-lives of the analyzed proteins.

The FSR calculation using equation (1) necessitates the analysis of amino acids labeling in specific tissues in order to determine true precursor labeling. As mentioned above, invasive tissue analysis limits the application of this technique mainly to animal studies and complicates its translation to clinical research. In order to circumvent the problems related to the measurement of intracellular amino acid labeling, we developed an algorithm for estimation of the precursor enrichment based on accessible body fluids [37]. The rationale is similar to those used for heavy water-based lipid turnover studies and based on the fact that the ^{2}H-labeling of body water represents the precursor enrichment. Thus, the precursor amino acid enrichment in equation (1) could be replaced with the total body water enrichment. However, since a product (analyzed peptide) incorporates multiple copies of ^{2}H, the denominator in equation (1) should take into account the asymptotic number of deuterium (N) incorporated into a peptide:

$$\text{FSR} = \text{slope of product labeling}/\left(E_{\text{water}}{}^{*}N\right) \quad (2)$$

where E_{water} is the steady-state enrichment of total body water and N is the asymptotic number of deuterium atoms incorporated into a peptide, which is calculated using a mathematical algorithm. Since the asymptotic labeling of a peptide is a function of total body water and the number of exchangeable hydrogen atoms [$E_{\text{peptide}} = f(E_{\text{water}}, N)$], when two of the three parameters are known, the third one can be calculated. Thus, N can be calculated using a simple algorithm based on experimental measurement of a peptide's labeling (E_{peptide}) and body water

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enrichment (E_{water}). For this purpose, the software models an isotopomer distribution of a peptide based on plasma ^2H_2O labeling and the different numbers of incorporated ^2H atoms and compares that with the experimentally measured plateau labeling of a peptide. The theoretical isotopic distribution is calculated based on the elemental composition of a peptide sequence and the number of incorporated ^2H atoms. Each calculated isotope distribution is then correlated against the measured isotopic distribution, and the best fit of *N* is determined based on the minimum of the sum of squares error between the theoretical isotopic distribution simulated by the program and the experimentally measured isotope distribution. Plasma ^2H_2O labeling is measured using an acetone exchange method, and the isotope distribution of a peptide(s) is determined using high-resolution full scan spectra. Thus, estimation of the FSR in a short-term experiment requires measurements of peptide labeling by LC-MS/MS, water labeling by GC-MS [48], and calculation of the asymptotic number of deuterium atoms incorporated into the peptide (i.e., the *N*) using a mathematical algorithm [37].

We demonstrated the utility of this approach by quantifying the effect of the nutritional status on the synthesis of albumin and other acute-phase response proteins in rats [15]. With this approach, protein turnover could be determined in a few hours with the total body water (TBW) enrichment of ~2.5%. For the plateau labeling of analyzed plasma proteins, we used the data from our 10-day ^2H_2O experiment. Since the half-life of rat albumin is ~1.8 day, the number of incorporated deuterium atoms from 10-day labeling experiment (i.e., 5 half-lives of albumin) is close to the maximum possible ^2H incorporation. This short-term 7-h ^2H_2O labeling protocol allows measurement of the kinetics of proteins with a wide range of rate constants (~1%/h for albumin and ~16%/h for ApoB100). Calculated half-lives of different plasma proteins observed using this approach agree with their known biological functions. For example, rapid FSRs were observed for the acute-phase response proteins haptoglobin and fibrinogen. Hemoglobin, albumin, and ApoAI which are involved in oxygen delivery, fatty acid transport, and reverse cholesterol transport, respectively, have the longest half-lives from all the studied plasma proteins. The observed half-lives are also in agreement with the *N*-end rule, which states that the half-life of a protein is determined by the nature of its *N*-terminal amino acid residue [49]. ApoB, ApoE, and haptoglobin with destabilizing amino-terminal Phe, Gln, and Asn, respectively, have shorter half-lives, while hemoglobin, albumin, ApoAI, and ceruloplasmin with Ala (albumin and ApoA I) and Gly (ceruloplasmin) have longer half-lives.

A short-term (e.g., 7-h) ^2H_2O-labeling experiment in rats also allows assessing the effect of nutritional status on the synthesis of plasma proteins, including albumin. Using this approach, it was determined that fasting has a divergent effect on protein synthesis in accordance with the biological function of the protein. In agreement with previous studies using amino acid tracers, it was found that fasting increases the synthesis rate of ApoB100 while reducing the synthesis rates of albumin and fibrinogen. Stimulated synthesis of ApoB100, the principal protein of very-low-density lipoprotein (VLDL), suggests increased secretion of VLDL, a well-known phenomenon in fasting. However, the synthesis rate of albumin, the most abundant plasma protein, was reduced ~twofold in the fasting state as compared to the fed state. Presumably, this was related to the regulation of albumin synthesis by amino acid substrate availability.

2.2. The long-term heavy water protocol for protein synthesis

Although the short-term experimental design enables one to assess the turnover rates of plasma proteins in several hours, it requires the knowledge of the precursor enrichment. Alternatively, a long-term labeling protocol allows one to measure protein turnover based on modeling of the time-course labeling of analyzed peptides without knowledge of precursor enrichment; note that this is often based on the assumption of a single compartment [15, 22]. The drawback of this design is that it requires the collection of multiple samples for the curve fitting. The FSR in a long-term experiment is calculated by fitting the time-course total labeling of a peptide ($E_{\text{peptide}}(t)$) to an exponential rise curve equation:

$$E_{\text{peptide}}(t) = E_0 * \left(1 - e^{-kt}\right) \tag{3}$$

where E_0 is the calculated asymptotical total labeling and k is the rate constant. An accurate calculation of the rate constant requires at least five appropriately timed data points, and greatly depends on the accuracy of the last time-point measurement. Ideally, it is preferred that the last time point corresponds to asymptotical labeling; however, the presence of sufficient early time points will also accurately predict the theoretical E_0. The half-life of a protein is determined based on the turnover rate constant: $t_{1/2} = \ln 2/k$.

Total labeling of a peptide will be calculated using the formula:

$$\text{MPE} = \text{MPE}M_1x1 + \text{MPE}M_2x2 + \ldots + \text{MPE}M_ixi \tag{4}$$

where MPE M_i is the molar percent enrichment of an isotopomer and calculated as

$$M_i = \left(M_i / \Sigma\left(M_0, \ldots M_i\right)\right) * 100\%) \tag{5}$$

Similar to other tracer experiments, there is a time delay between ^2H_2O administration and the effective onset of a protein labeling. Such delays most likely reflect a lag between ribosomal protein synthesis and export. Secretory proteins are synthesized on polysomes bound to rough endoplasmic reticulum (ER) and are transported to the lumen of the ER. Before secretion, proteins are transported from the ER to the Golgi apparatus and there is a temporal delay in the transfer from the ER. This delay is especially important in calculation of FSR for relatively fast turnover proteins, such as ApoB100 [50]. It takes ~30 min for newly synthesized ApoB100 to be packaged and released into the circulation; thus, there is a time lag between protein synthesis and appearance in the plasma. To take the delay into account, the expression of $E_{\text{peptide}}(t)$ must be modified for an accurate calculation of the rate constant:

$$E_{\text{peptide}}(t) = E_0 * \left(1 - e^{-k(t-\tau}\right) \tag{6}$$

where τ is the delay time.

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In both short-term and long-term heavy water metabolic labeling experiments, the production rates (PR) for a protein is calculated as the product of FSR and the respective pool size of a given protein:

$$\mathrm{PR}\left(\mathrm{g}\times\mathrm{kg}^{-1}\times\mathrm{h}^{-1}\right) = \text{pool size} \times \mathrm{FSR} \tag{7}$$

where the pool size is an absolute content of a protein. In the case of plasma proteins, the pool size is the product of a protein concentration and plasma volume, estimated as 45 ml/kg body weight. Plasma concentration of a protein can be measured using a standard enzyme-linked immunosorbent assay (ELISA) techniques or the isotope dilution method by mass spectrometry [51].

Although the low dose of ^2H_2O (~0.5% TBW enrichment) is well tolerated in humans, the transient dizziness has been observed in some subjects with the higher bolus aiming to bring TBW enrichment 1.5–2% [52]. To reach this high level of ^2H_2O, according to the original study designs, human subjects ingested 4–5 smaller doses of ^2H_2O over 4–5 h. Recently, instead of a primed bolus, the gradual increase of ^2H_2O of TBW enrichment was proposed. According to this protocol, ^2H_2O enrichment of TBW exponentially increases and reaches the plateau value [25, 53]. The gradual increase of ^2H_2O in body fluids prevents any side effects related to ^2H-isotope effect. This nonsteady-state labeling of TBW increases the study duration and somewhat complicates the calculation. We applied this approach, i.e., slow increase of ^2H_2O enrichment of TBW, to study mitochondrial proteome dynamics in a rat model of heart failure [54]. We also constructed a new algorithm to calculate the time-dependent changes in heavy mass isotopomers of newly synthesized peptides. To account for the relatively slower increase in body water labeling, we fit the measured body water enrichment into an exponential curve that yields the body water turnover curve. Then, the modeled continuous body water curve was used for estimation of kinetically relevant body water enrichment required for accurate calculation of synthesis rates. We demonstrated that the calculated turnover rate constants for mitochondrial proteins using this nonsteady-state labeling protocol are very similar to those based on the steady-state bolus labeling of TBW [55]. Thus, this data analysis approach allows accurate quantification of the rate constants to analyze a protein turnover when ^2H_2O is administered without a priming bolus. This is of particular importance for human studies when it is preferable to increase the TBW enrichment gradually in order to eliminate concerns related to occasional transient dizziness observed with a high bolus dose of heavy water [52]. This also simplifies the study design, since small amounts of heavy water can be consumed outside of the clinical research unit without interference with the daily lifestyle of study subjects.

3. High-resolution mass spectrometry for heavy water-based proteome dynamics studies

Like other stable isotope-based turnover studies, heavy water metabolic labeling requires sensitive and reproducible measurements of isotope labeling of proteins. This necessitates an

accurate quantification of isotopomer distribution of protein-bound amino acids or tryptic peptides unique to a specific protein. Accurate and precise estimates of the isotopic ratio are critical when one aims to quantify subtle changes in protein synthesis due to diseases or an intervention.

Classical studies of protein turnover studies with heavy water utilized GC-MS to measure ^{2}H-incorporation into protein-bound amino acids after the hydrolysis of protein(s). Because of the low cost of GC-MS instruments, they have traditionally been more accessible than LC-MS instruments. In addition, until recent developments in high-resolution ion detections, many LC-MS instruments had lower accuracy in isotope ratio measurements compared to simple GC-MS instruments. A gas chromatography inlet enables separation of individual amino acids and quadrupole mass analyzer allows accurate measurement of isotope enrichment with ± 0.3%. In the case of ^{2}H-labeled compounds, heavy isotopomers enriched with ^{2}H are slightly shifted and eluted in front of the monoisotopic signal (M0). This chromatographic fractionation was used for the accurate quantification of low ^{2}H enrichment in amino acids and other molecules. With this approach, as low as 0.01% ^{2}H could be accurately measured using a simple quadrupole GC-MS instrument [56]. The majority of early studies with GC-MS were focused on total body or tissue-specific mixed protein turnover without giving knowledge about individual proteins. Later on, this approach was extended to the analysis of purified individual proteins. This requires labor-intensive purification of individual proteins and permits only analysis of one protein at a time. In addition to being time consuming, these protocols suffer from potential contamination associated with protein isolation. The development of isotope-ratio mass spectrometry (IRMS) systems adds more than 100-fold increase in sensitivity for measuring of ^{2}H enrichment compared with GC-MS [57]. However, similar to GC-MS, IRMS instruments are limited to analysis of protein-bound amino acids.

Recently, a proteomics-based approach was applied to assess the protein turnover in a mixture of proteins [15, 25, 47]. In contrast to static proteomics, the dynamic proteomics method requires accurate quantification of the isotope distribution of peptides that requires high-resolution mass analysis. Studies by Anderson's group evaluated the utility of different type of electron spray ionization (ESI) and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry for the isotope distribution analysis [58, 59]. A Finnigan TSQ 700 or Micromas Quatto II, Thermo-Finnigan linear trap quadrupole (LTQ) linear ion-trap and Applied Biosysytems Q-STAR XL hybrid quadrupole-TOF, and Bruker BiFlex III MALDI-TOF were tested [59]. Tandem spectra on the ion-trap instrument were collected in either a zoom scan or profile mode while the quadrupole instrument was operated in the selected ion monitoring (SIM) mode. It has been determined that the signal intensity is the key parameter for accurate characterization of isotope distribution. For instance, the quantification of M1 with precision better than 5% requires intensities of the base peak ≥20,000 counts in a MALDI-TOF instrument. Based on our experience, similar precision on LTQ linear ion-trap instrument can be achieved with an ion intensity of 10^{4} relative to the background signal. It has been noted that MALDI-TOF slightly overestimates M1.When the ESI trap and quadrupole instruments were tested for the accuracy and precision of isotope distribution, the ion-trap MS performed better than the SIM quadrupole MS. Interestingly, the quadrupole instrument in SIM mode

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had greater precision than MALDI-TOF MS and the accuracy of the quadrupole measurement was improved when it was operated in a profile scan mode.

When applied to protein turnover studies, the high resolution of MALDI-TOF MS allows accurate quantification of ^{2}H enrichment of tryptic peptides [22]. The traditional proteomics methods coupled with MALDI-TOF MS-assisted isotope distribution analysis greatly advanced protein turnover studies in a mixture of proteins. However, the absence of a chromatographic inlet enables the analysis of only the most abundant proteins and therein compromises a broader application to low abundant proteins. Also, regardless of the peptide abundance, the presence of interfering signals compromises the utility of MALDI-TOF MS for turnover studies in a complex mixture of proteins. To avoid this issue, we also evaluated the utility of the linear ion-trap LTQ instrument for measurement of the fractional synthesis rates of plasma proteins [15]. The high sensitivity of LTQ MS in zoom scan mode allows accurate measurement of the kinetics of proteins and the assessment of changes in plasma proteins synthesis rates related to animal nutritional status. One of the limitations of this instrument is that only a limited number of peptides can be targeted in each duty cycle which is limited by the scan speed. To increase the number of analyzed proteins in a single run, the chromatogram can be divided into several time segments. In this case, several peptides are analyzed in each time segment. Still, this approach allows only the quantification of isotope distribution using 10–15 peptides from 3–6 proteins using 2-h high-performance liquid chromatography (HPLC) gradient and well-designed MS method. Although several mass spectrometer platforms with liquid chromatographic inlets allow accurate quantification of the isotope distribution, only the high-resolution mass spectrometers permit measuring protein turnover on a truly proteome-wide scale.

It has been shown that quadrupole time-of-flight (Q-TOF) MS instruments have a good reproducibility and can accurately measure isotope ratios [60], and it was utilized to study the lipoprotein turnover in mice. However, Q-TOF instruments have relatively lower resolution (~30,000) that limits the isotope ratio accuracy of the isotope ratio analysis. By contrast, the hybrid Fourier transform ion cyclotron (FT-ICR) and Orbitrap mass spectrometers are characterized by unsurpassed resolution (>100,000), high mass accuracy, and sensitivity [61, 62]. The high mass accuracy of these instruments improves identification and characterization of peptides, while high resolution provides additional information for the characterization of the molecular formula based on natural enrichment. Importantly, the high resolution of these instruments, coupled with the increased scan rates, allows accurate isotope distribution analysis that enables measurement of metabolic labeling of all analyzed peptides. Recently, we demonstrated that isotopic ratios between the monoisotopic and heavy isotopic peaks are consistently lower than predicted values and the magnitude of the spectral error in the FT-ICR MS is proportional to the scan duration of the ion clouds (i.e., resolution setting) [63]. It has been shown that the logarithm of the measured isotopic ratio linearly decreases with the acquisition time, and this phenomenon has previously been used to improve the accuracy of the isotopic distribution analyses [64]. However, even at the lowest resolution setting (e.g., 7,500) a significant error (~5%) was observed with FT-ICR MS analysis. Mass accuracy and isotopic ratios may be affected by the static Coulomb repulsion of ions, so fewer ion numbers

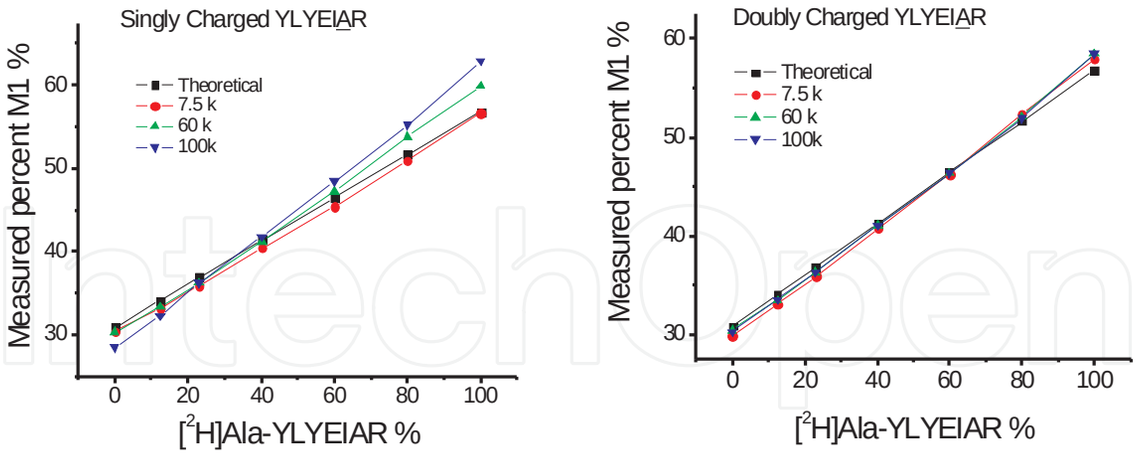

Figure 4. Calibration curves of [2-^{2}H]alanyl-YLYEIAR enrichment (0–97%) measured at different resolutions in Orbitrap Elite instrument. More than 5% error was observed at higher resolution (100 K) for a singly charged peptide ion. Measured enrichment of doubly charged peptide ion is similar to simulated theoretical values.

could reduce the error. However, accurate quantifications of isotopomers require a sufficient number of ions. We found that ion intensities could be accurately measured with ion counts ranging from ~10,000 to 100,000. In this range, the isotopic ratios are approximately the same, while higher ion counts leads to greater error in isotope ratio measurements. To obtain accurate isotopic ratio measurements of peptides, multiple scans with different durations were performed, and the data were extrapolated to the initial moment of the ion rotation. This approach minimizes the absolute isotopic ratio error to within ~1–0.5%. In addition, we found that monitoring the parent ions in the SIM mode (mass interval is 10 Da), and the collision-induced dissociation (CID) fragments in the single reaction monitoring (SRM) mode, improves the specificity of the assay and allows selective identification of peptide and its fragments for isotopomer analysis. Using SIM and SRM experiments in the same acquisition allows reliable simultaneous quantification of the isotopic distribution of both the parent peptide and its fragment ions [37]. An accurate measurement of two consecutive peptide fragments allows one to calculate the labeling of protein-bound amino acids, including alanine, glutamine, and glutamate [37].

Next, we tested the utilities of hybrid Orbitrap Velos and Orbitrap Elite instruments for the ^{2}H-based metabolic labeling studies [54, 55]. To evaluate the utility of the newer generation Orbitrap Elite instrument for isotope distribution analysis, a calibration curve was constructed by adding an increasing amount of [2-^{2}H]alanyl-YLYEIAR to a constant amount of unlabeled YLYEIAR. Interestingly, similar but lower magnitude error in isotope ratios was observed in both Orbitrap instruments. Consistent with previous studies [65], the Orbitrap also yields higher error at higher resolution setting. The Orbitrap Elite displayed the highest accurate isotope ratio measurements. We found consistent underestimation of the isotope ratio measurement when lower ^{2}H enrichment was measured, while overestimation was observed at the higher ^{2}H enrichment (Fig. 4). Interestingly, the error was less when doubly charged ions of the same peptide were analyzed.

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The accuracy and precision of molar percent enrichment (MPE) determinations, calculated as the fraction of the total intensity, depends on the number of isotopomers that are used in the calculation. This is due to a lower abundance of heavy isotopomers which introduce more error in MPE calculation. To circumvent this problem, an alternative approach, i.e., M1/M0 ratio, was proposed to assess ^{2}H-induced changes in an isotope distribution [59]. Although this approach is useful for the modeling of the labeling data in a long-term experiment, it does not allow one to assess the total labeling of an analyzed peptide and asymptotical number of ^{2}H, the critical step in calculation of the FSR in a short-term experiment.

4. Data analysis in global proteome dynamics studies with heavy water

The high-resolution mass spectrometers allow one to analyze isotopic distributions of virtually all peptides, thus enabling measurement of global proteome dynamics. The bottleneck in these experiments is the data processing. Therefore, high-throughput and robust bioinformatics tools are required to extract the relative isotopomer information from time-course data for the calculation of protein turnover rate constants based on large volume and complex data sets generated by high-resolution mass spectrometers.

Several software solutions have been proposed for the tracer-specific protein turnover studies. SILACtor has been successfully used for protein turnover SILAC experiments in cell culture [66]. SILACtor is useful for *in vitro* proteome dynamic experiments when the heavy precursor is 100% enriched and the protein product labeling gradually increases from 0 to 100%. However, it is not applicable to *in vivo* experiments when only partial labeling is feasible. The Topograph software developed by Macross and colleagues is another software that analyzes data from pre-labeled amino acid experiments, and it is applicable to both *in vitro* and *in vivo* experiments [67].

The heavy water-metabolic labeling approach poses further specific challenges to data analysis software [68]. In contrast to protein turnover studies with pre-labeled amino acids that lead to substantial average mass shifts in newly synthesized proteins, the labeling with heavy water mainly affects the relative isotopomer distribution without a measurable mass shift (maximum ~0.2–0.4 Da in an average mass of tryptic peptides). Thus, the partial labeling of proteins with the overlapping isotope profiles of labeled and unlabeled species complicates routine data analysis with $^{2}H_2O$-labeling approach.

Therefore, the successful implementation of the heavy water labeling experiment, in addition to improvements in mass spectrometry, sample preparation, and fractionation, depends on the efficiency of robust software for data processing. It is also preferable that the software could handle the data generated by different high-resolution instruments. Recently, several high-resolution mass spectrometer platforms have been used to study protein turnover using stable isotopes, including $^{2}H_2O$. Q-TOF mass spectrometer (Agilent) was applied to assess the proteome dynamics in plasma and different tissues [25]. For the data analysis, the authors used MassHunter software package (B0.4) from Agilent (Santa Clara, CA) specially designed for the isotopic distribution analysis of peptides processed in Agilent 6520 Q-TOF mass spec-

trometers. As Agilent's proprietary software, the MassHunter software package is not freely available to the public. Although this software facilitates the analysis of data generated in Agilent Q-TOF MS, for accurate isotopomer profiling, each sample is analyzed twice: during the first injection, MS/MS spectra are collected for protein identification and a second injection was performed for high-resolution full scan acquisition which doubles the instrument time per sample and limits high-throughput analysis. In addition, unlike high-resolution FT mass spectrometers, Q-TOF instruments have relatively lower resolution (~30,000 compared to 120,000 in Orbitrap Elite) that limits the accuracy of isotope ratio-based turnover measurement in this instrument.

Although currently available FT LTQ-ICR and LTQ-Orbitrap hybrid instruments allow both MS/MS scans and full scan analysis in a single acquisition with unsurpassed high resolution, in contrast to an Agilent Q-TOF instrument, they are not supported with software that could automatically extract the data from high-resolution full scan spectra. Thus, specialized software for automated high-resolution data analysis is critically needed. To advance $^{2}H_2O$-metabolic labeling for *in vivo* studies of protein turnover, the new software must be robust, user friendly, accurate, and capable of producing statistically rigorous results. Recently, Ping and colleagues described a software IsotoQuan/RateQuant–ProTurn [47] for calculation of peptide isotope distribution and protein turnover rates from heavy water labeling experiments. The software has been useful for processing data sets from $^{2}H_2O$-metabolic experiments. It uses manual validation for peak integration, and fixed exponential decay functions for protein turnover rates calculation.

In the original version, the software used a mass accuracy of 100 ppm and resolution of 15,000, which increases the likelihood of contamination of mass isotopomers by co-eluting signals. To avoid the complexity caused by co-elution, the mass spectrometers were operated at lower resolution (15,000) and mass accuracy (100 ppm) [69] which simply masks the interfering signals due to low resolution. A later version of the software included more stringent filtering parameters: a mass window of 75 ppm is recommended for 30,000 or 60,000 resolution (http:/www.heartproteome.org/proturn/index.html). However, this software is not freely available to the public, and the raw data from outside investigators could be processed only with the assistance of a web administrator. So far, to the best of our knowledge, no study from outside investigators has been reported using this software.

To aid our heavy water-based proteome dynamics studies, we recently developed an alternative software [55] which is available at the University of Texas Medical Branch (UTMB) website, https://ispace.utmb.edu/users/rgsadygo/Proteomics/HeavyWater/Version.1.0. Although this semiautomated software still requires a skilled operator for the data analysis, to the best of our knowledge, this is the only freely available software for quantification of proteome dynamics using heavy water-based metabolic labeling approach. With this software, a routine data analysis workflow for the heavy water labeled samples starts with the peptide/protein identifications from tandem mass spectra using protein sequence databases. Thus, the software reads all peptide IDs from the MASCOT mzIdentML files and confirms each ID based on the stored MS/MS scans at every time point. The initial step is to overlay the chromatographic profiles for each LC-MS run from all time points. Then, the software generates extracted ion

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chromatograms for each isotopomer for positively identified peptides from the high-resolution full MS scans within the elution time window of the corresponding MS/MS scan. In addition to peptide selection based on an exact mass and retention time, the software also filters unlabeled peptides at the baseline (t=0). For this purpose, theoretical masses are calculated as additional confirmation of a peptide's identity. Only peptides satisfying the modifiable filtering criteria based on exact mass (<10 ppm), peptide score (>35), signal intensity (>10^4), and peptides present in at least five time points of ^2H labeling are selected for quantification. The latter criterion is required to obtain sufficient data points for the kinetic modeling of the data. Although this conservative selection of peptides reduces the number of analyzed proteins, it substantially improves the accuracy of the results. The chromatographic profile of a peptide is determined by estimating the signal-to-noise ratio. The software removes peptide IDs that have chromatographic overlaps with other signals and a spectra of low quality (low signal-to-noise ratios or low MASCOT scores) based on the correlation of individual peaks across the elution profile. Also, peptides that cannot be assigned to a unique protein are excluded. All outliers are removed using appropriate statistical methods.

Next, the mass isotopic distributions for all selected peptides are quantified as a function of time. Peaks intensities are extracted from the averaged full scan by searching for an intensity that is maximum within the theoretical mass window (±10 ppm). We then use separate software to compute FSR, and the values for the same proteins are averaged. Examination of large data sets reveals that even with using these stringent criteria, contaminating signals may result in inaccurate rate constant calculations. This could be related to contributions from minor overlapping unresolved peaks that may not be easily filtered during isotope distribution analysis by the software. Therefore, a second-line filtering step of "contaminated peptides" involves the elimination of outlier peptides based on the coefficient of variation in the protein turnover rate constant relative to the average of the other peptides. Thus, the extracted data from only those peptides that could be modeled with the regression coefficient cutoff of 0.95 for nonlinear curve fitting and coefficient variation less than 30% relative to average of other peptides are selected for final quantification of the rate constants. These stringent selection criteria combined with precise isotope distribution analysis results in accurate quantification of protein synthesis rates.

5. Biological application of heavy water-based proteome turnover studies

Recent technological advancements in bioanalytical instrumentation and their application to systems biology are starting to significantly advance our understanding of integrative physiology. These achievements would not be possible without progresses made in genomics, proteomics, and metabolomics, that is, "omics" technologies that enable comprehensive screening of the genome, proteome, and metabolome [70, 71], respectively. The immense information collected using these "omics" sciences helps to understand the diseases mechanisms and facilitates early diagnosis of the disease, along with implementation and evaluation of personalized therapy [72]. Utilization of genomics in particular enabled the discovery of several genetic diseases. Although multiple protein biomarkers have been identified using

quantitative proteomics, compared to genomics, proteomics is still lagging behind as a clinical test method. This is partly related to the complexity of the human proteome. In addition, profiling of proteins may not be sufficient to understand physiological changes in a living organism, because they have inherent limitations associated with the low sensitivity of static measurements which are the end result of the changes in dynamic flux. In general, stress-induced changes in a biological system first affect the flux of a protein(s) that may lead to more drastic changes in their pool sizes. Only an uncompensated response to stress would result in the nonsteady-state changes in the synthesis or degradation of proteins leading to alterations of their pool sizes. Importantly, the magnitude of changes in flux measured with a small amount of tracer often exceeds the changes in large pool sizes. This is why the kinetic measurements are usually more sensitive than static measurements. In addition, if the stress equally increases or decreases both synthesis and degradation, then the pool size may not change at all. Isotope-based technologies allow investigators to measure changes in flux, and recently, "fluxomics" joined the "omics" sciences. Stable isotope-assisted dynamic metabolomics helped discover previously unknown metabolic pathways [73]. While fluxomics measures large numbers of metabolite turnover, a stable isotope-assisted protein turnover investigates the dynamic genome expression through the temporal changes in a protein flux. Thus, the traditional static proteomics, coupled with a metabolic labeling approach and high-resolution mass spectrometry, is expected to provide a means for simultaneous measurements of proteome dynamics. From the tracer selection point of view, a heavy water-based metabolic labeling approach is of particular interest. For example, H is the ubiquitous element of all biological molecules, and as a universal tracer, ^2H_2O labels DNA, RNA, proteins, and metabolites and provides the wealth of information in integrated comprehensive "omics." Because of our focus on proteomics, we will mainly highlight the biological application of ^2H_2O-based proteome dynamics.

Since proteins are indispensable to life activity and involved in multiple structural functions, enzymatic, activities, signal transduction, growth, and repair functions, only minor alterations in a protein homeostasis can lead to genetic and acquired diseases. Mass spectrometry-based protein turnover studies enables the analysis of perturbations in the protein metabolism in different diseases. Recently, ^2H_2O-based metabolic labeling approach was applied to study proteome dynamics in whole blood, blood cell fractions, plasma, whole tissue samples, and cell organelles. Here we will focus mainly on *in vivo* animal and human studies with ^2H_2O.

5.1. Animal studies

Early studies with the ^2H_2O-based labeling approach were focused on plasma albumin and mixed tissue proteins synthesis. Using a rat model, it was found that plasma protein synthesis is very sensitive to nutrient availability and ~50% of plasma albumin that was synthesized over a 24-h period was produced within ~5 h after the meal [74]. Furthermore, this study demonstrated that the heavy water approach also permits the analysis of plasma albumin synthesis during metabolic "steady-state" and "nonsteady-state" conditions corresponding to fasted and fed states. Consistent with these results, using a proteomic approach, we demonstrated albumin synthesis in rats was significantly reduced after 22-h fasting [15].

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The effects of dietary factors on tissue protein synthesis were investigated in acute fasting (20 h) vs. chronic food restriction (7 days), and feeding (a single meal) conditions in rats. Both acute and chronic fasting significantly reduced mixed tissue protein synthesis in the liver and gastrocnemius muscle, while it did not affect protein synthesis in the left ventricle of the heart [32], indicating that cardiac protein synthesis is preserved in conditions of nutritional perturbations. The follow-up studies demonstrated that diet-induced obesity in mice did not affect the skeletal muscle protein synthesis; however, it did impair the response of muscle protein synthesis to nutrient supply [34].

Understanding the mitochondrial proteome is a new emerging area in proteomics analysis which is largely aimed at targeting over one thousand proteins that are critical in adenosine triphosphate (ATP) synthesis and cell signaling [75]. Mitochondrial dysfunction plays a key role in aging and different diseases associated with oxidative stress and impaired energy metabolism [54, 76]. Therefore, recent attention toward mitochondrial biogenesis [77, 78] and proteome dynamics [55, 69] became an intense area of research in mitochondrial biology. The wide range of concentration of mitochondrial proteins poses a great challenge for comprehensive analysis of the mitochondrial proteome. Nevertheless, several fractionation and enrichment methods have been used to map mitochondrial proteomes. Different labeling approaches have been applied to measure the turnover rates of mitochondrial proteins. For example, [$^2H_3$]-leucine was used to assess the *in vivo* turnover rates of mitochondrial proteome in the mouse liver and heart [67]. We utilized the ^2H_2O-based metabolic labeling technique to assess protein kinetics in cardiac, brain, and liver mitochondria. Adult rats were given ^2H_2O in the drinking water for up to 60 days. Plasma ^2H_2O and myocardial and hepatic tissue ^2H enrichment of amino acids were stable throughout the experimental protocol [55]. Analysis of mitochondrial protein synthesis in rat liver revealed that the half-lives of proteins range from 2 to 6 days. In the heart, the two spatially distinct subpopulations of cardiac mitochondria, subsarcolemmal (SSM, found along the perimeter of the cell) and interfibrillar (IFM, located between the myofibrils) mitochondria, were analyzed. It is well known that SSM and IFM populations have distinct biochemical functions with IFM having a greater respiratory capacity and resistance to Ca^{2+}-induced stress [79, 80]. Multiple tryptic peptides were identified from each protein in both SSM and IFM, and showed time-dependent increases in heavy mass isotopomers that was consistent within a given protein. In contrast to the liver, cardiac mitochondrial protein synthesis was relatively slow (average half-life of 30 days, or 2.4% newly made per day). Thus, the rate of synthesis of cardiac mitochondrial proteins is approximately sevenfold longer than that of the liver. Analysis of protein synthesis based on protein location within the mitochondrion revealed a shorter half-life for outer membrane proteins than inner matrix proteins in both SSM and IFM. Subunits of mitochondrial electron transport chain (ETC) complexes and proteins with other related functions displayed similar half-lives, suggesting that the differences in mitochondrial proteins turnover could be explained by their subcomplex association. Although the synthesis rates for individual proteins were correlated between IFM and SSM (R^2=0.84, p<0.0001), values in IFM were 15% less than SSM (p<0.001) [55]. The differences in distinct mitochondria populations may have a particular relevance to mitochondrial dysfunction in different diseases, since previous studies found differential effects of aging, diabetes, and heart failure in SSM and IFM. It has been shown that IFM are

more susceptible than SSM to disease-associated damage [81]. In particular, rats with advanced pressure overload-induced heart failure have greater dysfunction in IFM than SSM, suggesting severe impairment in protein synthesis and/or stability in IFM than in SSM.

Previously, it has been shown that the turnover rate of the total mixed mitochondrial brain proteins are slower than those of cardiac proteins [82]. When we compared the turnover rates of individual proteins in the rat brain and heart mitochondria, we found that in the brain, the turnover rate of superoxide dismutase is indeed slower than in the heart (Fig. 5). By contrast, ATP synthase F1β has a much faster turnover rate in the brain than the heart, suggesting that the kinetics of individual proteins in each organ is determined by their functions. Consistent with previous studies [82], we found that similar to the heart, the turnover rates of all analyzed mitochondrial brain proteins had much slower turnover rates compared to those in the liver.

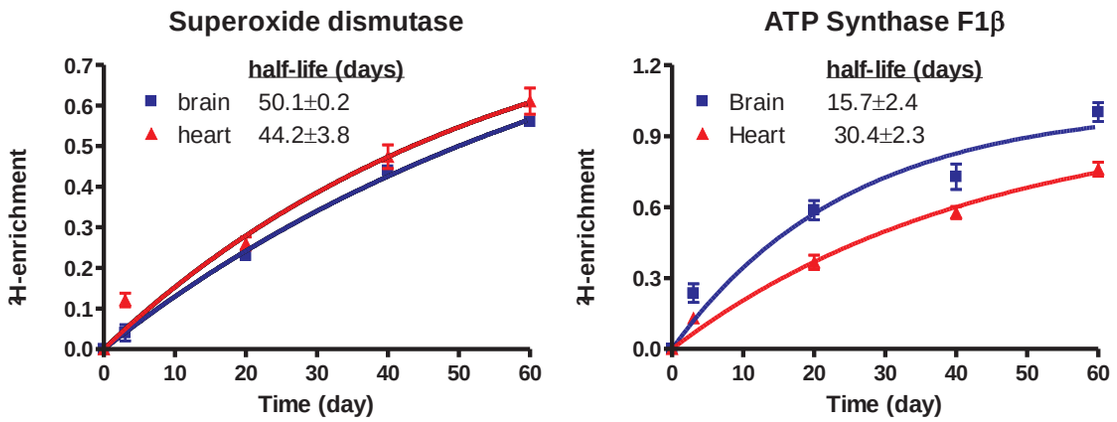

Figure 5. Comparison of half-lives of brain and heart mitochondrial proteins.

To test the effect of heart failure on the stability of cardiac mitochondrial proteins, we utilized our ^2H_2O approach to measure mitochondrial proteome dynamics in a well-established rat model of heart failure induced by chronic transverse aortic constriction (TAC) [54]. Decreased mitochondrial ATP generating capacity in myocardium is a hallmark of heart failure; however, the underlying mechanisms contributing to mitochondrial dysfunction in heart failure are not yet fully understood. Rats with TAC develop moderate heart failure after 22 weeks, which results in left ventricular remodeling, dysfunction, and reduced oxidative capacity in mitochondria. Heart failure caused a decrease of mitochondrial proteins and respiratory capacity in IFM, but not in SSM. We used a heavy water method to determine whether the decreased synthesis of mitochondrial proteins contribute to the respiratory dysfunction in heart failure. Although the synthesis rates of proteins in IFM tend to be higher than those in SSM, it only started to reach modest significance (p=0.08) in this study. Surprisingly, in spite of the changes in the mitochondrial protein content, the average rate of protein synthesis (based on the kinetics of 49 proteins with different functions) was similar in sham-treated and heart failure groups. This was due to bidirectional changes in the synthesis rate of different mitochondrial proteins. In particular, heart failure increased the turnover rate of several proteins involved in

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fatty acid oxidation, electron transport chain, and ATP synthesis, while it decreased the turnover of other proteins, including pyruvate dehydrogenase subunit in IFM, but not in SSM. The study of proteome dynamics suggested that reduced respiratory capacity in IFM might be related to increased degradation of several IFM proteins involved in fatty acid oxidation and ETC. Interestingly, proteins with destabilizing *N*-terminal amino acids of mature proteins exhibited shorter half-lives compared to those with stabilizing *N*-terminal amino acids.

Thus, the kinetic measurements of mitochondrial proteins may help understand the mechanisms responsible for mitochondrial alterations in the failing heart. Taken together, utilization of the ^2H_2O method for mitochondrial proteome studies demonstrated that this method is robust and can distinguish subtle differences in synthetic rates between subcellular populations of mitochondria. In addition, measuring the kinetics of individual proteins enables one to uncover changes in the mitochondrial proteome due to heart disease that cannot be obtained by simply measuring their static expression at any given time point.

In a follow-up study, Lam and coworkers applied the heavy water method to determine protein kinetic signatures of β-adregengic-induced cardiac remodeling in a mouse model [47]. Several kinetic markers of calcium signaling, energy metabolism, proteostasis, and mitochondrial dynamics were identified. Although large set of data was generated, the biological relevance of these results requires further evaluation based on protein properties and pathways that they are involved.

Hellerstein and coworkers used ^2H_2O labeling-based dynamic proteomics combined with the stable isotope labeling in mammals (SILAM) quantitative proteomics to explain the effect of long-term calorie restriction on longevity [83]. Through assessment of both catabolic rate and absolute synthesis of hepatic proteins, the authors demonstrated that calorie restriction reduces the turnover of most (~80%) hepatic proteins, including mitochondrial proteins. Thus, long-term calorie restriction increases the stability of proteins and reduces global protein synthetic burden that is associated with decreased mitochondrial biogenesis and mitophagy. The pathway analysis revealed that proteins with related functions display coordinated changes. *In silico* analysis identified peroxisome profilator-activated receptor gamma coactivator 1-alfa as a potential regulator of altered network dynamics.

The ^2H_2O-labeling methods were also applied to identify kinetic biomarkers of neuronal dysfunction in mouse models of neurodegeneration [84]. After a bolus administration of ^2H_2O, appearance of ^2H-labeled neuronal proteins with transport and cargo functions in cerebrospinal fluid was quantified. Compared to controls, the appearance of proteins in mice with neurodegeneration was delayed, which was normalized after microtubule-modulating pharmacotherapy, suggesting that the transport kinetics may provide a test method for monitoring disease progression and therapy for neurodegenerative diseases.

We applied ^2H_2O-based metabolic labeling approach to assess the high-density lipoprotein (HDL) proteome dynamics in a diet-induced mouse model of nonalcoholic fatty liver disease (NAFLD) [85]. HDL displays multiple functions that include reverse cholesterol transport (RCT), preventing inflammation, oxidation, platelet activation, and maintaining endothelial function. In metabolic diseases associated with insulin resistance, HDL may lose these protective functions and become dysfunctional. The reasons for these changes are not fully

understood and may be attributed to alterations of the HDL particle composition and modifications of HDL proteins. In addition to ApoAI and ApoAII (which account ~65% and ~15% of HDL protein mass, respectively), recently more than 50 less abundant HDL proteins have been identified. These HDL proteins involved in lipid metabolism, acute-phase response, innate immunity, protease inhibition, and regulation of endothelial cell apoptosis that determines HDL's anti-inflammatory, anti-atherogenic, and cell survival properties. Thus, alterations in the HDL proteome composition may be a key factor involved in HDL dysfunction.

It is well known that a Western diet (WD, high-fat diet containing cholesterol) for 12 weeks leads to insulin resistance, NAFLD (hepatic steatosis, oxidative stress, and inflammation), and atherosclerosis (aortic root lesion) in low-density lipoprotein receptor ($LDLR^{-/-}$) mice. Proteomics analysis of ApoB-depleted plasma revealed that a WD also altered the levels of multiple proteins known to be associated with HDL. The kinetics of 60 previously identified HDL proteins involved in lipid metabolism, thrombosis, protease inhibition, complement regulation, and acute-phase response were quantified. The analyzed HDL proteins exhibited a wide range of half-lives varying from a few hours to days. For instance, in a standard chow diet-fed $LDLR^{-/-}$ mice, ApoE, ApoAI, and PON1 have half-lives 5, 15, and 64 h, respectively. A WD has differential effects on the turnover rates of proteins with different functions. We found that a WD results in decreased levels and increased catabolism of PON1 which is responsible for the antioxidant function of HDL. Interestingly, a WD also resulted in increased levels and turnover of phospholipid transfer protein (PLTP), which is responsible for promoting HDL remodeling through phospholipid transfer from ApoB-containing particles to HDL. Mice deficient in PLTP are protected from atherosclerosis, while HDL from mice over expressing PLTP is dysfunctional in promoting cholesterol efflux, and these mice developed higher atherosclerotic lesion compared to control mice. Thus, ^2H_2O labeling allows to measure HDL proteome flux that is relevant to HDL functionality.

Since the RCT function of HDL represents the dynamic flux of cholesterol from peripheral tissues, including macrophage transfer to liver for clearance, we next applied our ^2H_2O-metabolic labeling approach to assess HDL flux as an *in vivo* index of RCT [31]. Because ^2H from ^2H_2O incorporates into both lipids and proteins, ^2H_2O allows studying the kinetics of both HDL-cholesterol (HDLc) and apoAI, the principal protein of HDL. Mice were given ^2H_2O in the drinking water and serial blood samples were collected at different time points. Fractional catabolic rates (FCR) for HDLc and apoAI were assessed based on their ^2H_2O-metabolic labeling. In addition, the synthetic heavy peptide of apoAI (VAPL($^6C_{13}$)GAEL($^6C_{13}$)QESAR) and [$^2H_6$]cholesterol were used for absolute quantification of pool sizes and production rates (PR) of apoAI and HDLc, respectively. $ApoE^{-/-}$ mice, which are prone to atherosclerosis, displayed an increased FCR (p<0.01) and a reduced PR of both HDLc and apoAI (p<0.05) compared to controls. In human apoAI transgenic mice (resistant to atherosclerosis), PRs of HDLc and human apoAI were strikingly higher than in wild-type mice. We also validated our HDL turnover method as an index of RCT. For this purpose, HDL turnover and macrophage-specific RCT were assessed in the same animals. Myriocin, an inhibitor of sphingolipid synthesis, was used as a modifier of HDL metabolism. Myriocin significantly increased HDL flux and macrophage-to-feces RCT, indicating compatibility of these methods. We conclude

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that ^2H_2O labeling can be used to measure HDLc and apoAI flux *in vivo*, and to assess the role of genetic and pharmacological interventions on HDL turnover.

^2H_2O labeling-based HDL turnover method also was applied to assess the effect of different isoforms of apoAI and gender on *in vivo* HDL function in wild-type human transgenic apoAI mice and mice with 4WF isoform of human apoAI, in which 4 tryptophan residues are substituted with phenylalanine [86]. The *in vitro* cholesterol efflux assay demonstrated that the 4WF isoform of apoAI was resistant to myeloperoxidase-induced loss of function while human apoA1 transgenic HDL lost all ABCA1-dependent cholesterol acceptor activity. This was associated with a small, nonsignificant increase in HDL turnover *in vivo*. Male mice displayed significantly higher plasma apoA1 levels than females for both isoforms of human apoA1, ascribed to increased production rate of HDL. Safety, simplicity, and low cost of the ^2H_2O suggest that this approach can be used for human use to study the effects of HDL-targeted therapies on both HDL proteome and HDLc dynamics.

5.2. Human studies

Although ^2H_2O has been used for more than 60 years in animal studies to measure a proteins' renewal rate, only in 2004 it was introduced to study protein synthesis rates in humans [87]. This first human study validated the basic underlying assumptions of ^2H_2O use in humans, i.e., equilibrium with total body water and amino acids is rapid and body water enrichment can be maintained constant for a long period of time. With ~0.4% TBW enrichment, the FSR of albumin based on albumin-bound alanine enrichment was determined to be ~4%/day in renal patients.

A recent study evaluated the long-term safety and hemodynamic effects of higher levels of heavy water ingestion in healthy young human subjects [53]. Subjects consumed 70% enriched ^2H_2O in 4 boluses of 0.51 ml/kg body weight daily during the first week of labeling. During the second week, the subjects consumed 4 boluses of 0.56 ml/kg. This protocol resulted in gradual increase of body water enrichment up to ~2% during the 14 days of heavy water exposure. The subjects' vital signs were monitored during ^2H_2O administration, and these subjects were followed up to an 8-month period. Total body water enrichment during exposure and subsequent physiological clearance from body fluids were determined during the following 2 weeks. No signs of discomfort and physiological effect were reported in these healthy young adults. After depletion of 14 of the most abundant proteins by multiple affinity columns, the tryptic digest from remaining proteins was fractionated using two-dimensional liquid chromatography separations and analyzed by the LTQ Orbitrap instrument. The turnover rates of hundreds of proteins were then determined. There was no correlation between protein turnover rates and protein abundance. Although many proteins involved in cardiovascular disease were also quantified, this proof of the concept study did not evaluate any link between protein turnover rates and disease. It was concluded that ^2H_2O is safe and effective tracer for large-scale human studies.

Several human studies utilized low-dose heavy water to assess the effect of exercise and cachexia on muscle protein synthesis. Gaiser and colleagues applied ^2H_2O (~0.3% TBW ^2H_2O enrichment) with a single biopsy protocol to test the effect of short-term (24-h) exercise on mixed muscle protein synthesis [46]. With this approach, the effect of acute resistance exercise

on integrative myofibrillar protein synthesis in healthy young subjects was determined. Subjects performed unilateral exercise using one leg while the other leg served as a control. Interestingly, exercise did not have any effect on the FSR of mixed muscle proteins. The high-intensity resistance exercise increased myofibrillar protein synthesis in the exercising leg (0.94±0.16%/h) compared to the control leg (0.75±0.08%/h, $p<0.05$), demonstrating that short-term low-level ^2H_2O exposure allows one to detect subtle changes in human muscle protein synthesis.

Recently, Wilkinson and colleagues expanded on these studies and investigated the effect of long-term (8-day) exercise on mixed muscle protein synthesis with heavy water for monitoring day-to-day changes in muscle subfractions (myofibrillar, collagen, sarcoplasmic) synthesis [88]. Similar to the study by Gaiser and colleagues, the authors employed a one-legged resistance exercise that allows use of the second leg as an internal control. The longer period of exercise and heavy water administration with multiple muscle biopsies at different time points in this study allowed them assess the changes in muscle protein synthesis in response to the temporal and cumulative successive bouts of exercise. By using the highly sensitive IRMS instrument, this study validated the utility of low dose (0.16–0.24% enrichment of TBW) heavy water for quantification of diurnal changes in muscle protein synthesis and for the assessment of short-term changes in protein turnover. It was demonstrated that protein synthesis in myofibrillar and collagen fractions was increased due to both short-term and long-term exercise; however, sarcoplasmic protein synthesis remained unchanged.

Scalzo and colleagues applied heavy water-based dynamic proteomics to assess integrated and individual muscle protein synthesis response and mitochondrial biogenesis for endurance exercise in males and females after 3 weeks of sprint interval training [89]. This study utilized 3 weeks of ^2H_2O-labeling protocol to achieve 1–2% TBW enrichment. It was demonstrated that due to exercise, muscle protein synthesis increased and the magnitude of change was higher in males compared with females. The increase in integrative muscle protein synthesis was associated with increased mitochondrial biogenesis assessed based on the synthesis rates of individual mitochondrial proteins and mitochondrial biogenesis signaling. It is important to note that it is unfeasible to use pre-labeled amino acid tracers for this kind of long-term studies of muscle protein synthesis, because this would require inpatient tracer infusion for several days.

Recently, a few studies utilized the heavy water method to assess the protein turnover in different diseases. A single oral dose of heavy water was applied to assess muscle protein synthesis in patients undergoing surgery for upper gastrointestinal cancer [90]. It was demonstrated that the mixed muscle protein synthesis was not decreased, rather, it was marginally increased as compared to healthy controls ($p=0.03$), suggesting that an increase in muscle breakdown may account for muscle wasting in cancer patients.

Studies from Hellerstein's group tested the utility of the heavy water method as a diagnostic tool in patients with psoriasis diseases [91]. The epidermal kinetics was determined in patients with psoriasis using twice-daily doses of ^2H_2O for 16–38 days. Keratin turnover was significantly accelerated in psoriatic lesions, suggesting that keratin synthesis could be used as a kinetic biomarker of psoriasis and other skin diseases.

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These studies demonstrated that the heavy water method has a great potential for human studies.

6. Challenges and future directions

Since ^2H_2O can be administered to humans, the dynamic proteomics approach could be widely used for clinical studies. Proteomics centers and infrastructure, which are equipped with state-of-the-art instrumentations and bioinformatics, exist in many areas in the USA and around the world. Static quantitative proteomics is already making highlights in clinical research and patient care. It is expected that in the near future, ^2H_2O will complement the traditional proteomics and expand to different areas of clinical research. The most obvious application of the heavy water method would be its utilization for the assessment of dynamics of circulatory proteins. Because of the high sensitivity of existing mass spectrometers, dynamic proteome analysis using small-tissue biopsy samples is also feasible. Thus, there is a great potential of using "dynamic markers" of health and disease. However, despite the wide-range potential for use in clinical settings, the heavy water method is still lagging behind as a diagnostic tool in patient care. This is partly related to several unmet methodological, instrumental, and bioinformatics challenges associated with studies of heavy water-based proteome dynamics. Unresolved issues related to the patient-oriented test design, user-friendly software development, and challenges centered around the data interpretation currently impede the routine clinical application of this technology.

In particular, a simple study design with a minimal number of short-term samples is very critical. This also requires creation of a reference database with human protein half-lives for implementation of a simple test for the proteins of interest based on their expected half-life ranges. In terms of methodological issues, still there are no published study on the effect of posttranslational modification and damage-induced aggregation of proteins on protein turnover and stability.

Although the mass spectrometry-based hardware tools are developing very fast, the cost of existing instruments is not easily affordable for many clinical laboratories which drives the cost of any proteomics test. Therefore, the cost reduction in this direction would facilitate the dynamic proteomics application as a clinical test method.

Some additional challenges are related to data interpretation and software issues. To advance *in vivo* studies of proteome dynamics with heavy water, high-throughput, user-friendly, robust, vendor-independent, accurate software capable of producing statistically rigorous results is needed. As mentioned in previous sections, currently there is no freely available software for comprehensive proteome dynamics data analysis. Although our recent software allows high-throughput data analysis, there are still several unmet bioinformatics challenges related to heavy water data analysis. One of the technical issues is related to quality control in data analysis. Sample complexity is the major challenge for automated data analysis. Although off-line liquid chromatography and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) (both 1D and 2D) separations simplify peptide mixtures, co-elution of peptides persists, even after the long-gradient chromatographic separation. This problem is

more severe with heavy water metabolic labeling, because in contrast to other tracers, ^2H_2O-metabolic labeling does not result in sizeable mass shifts of newly synthesized peptides, rather it leads to redistribution of incorporated ^2H among all heavy isotopomers. Therefore, it is critical to measure mass isotopomer distributions with the maximum number of heavy isotopomers. Although a simplified approach with M1/M0 has been proposed for the quantification of relative isotopomer abundances, an accurate evaluation of peptides ^2H enrichment requires tracing several isotopomers. Inclusion of all heavy isotopomers into calculations increases the chances of contamination by co-eluting species and chromatographic overlap signals. Although high mass resolution and accuracy substantially reduce the problem, sample complexity dramatically affects the turnover rate measurements, if not taken into account. Thus, the success in the computing of accurate turnover rates will depend on the availability of robust, easy-to-use software and bioinformatics tools for data analysis which would allow processing the co-elution profiles and extracting the mass profiles of the target species.

Our current software allows assessing the fractional catabolic and synthesis rates of a protein in a steady state. However, it is also critically important to know the absolute production rate of a protein and to determine whether protein abundance is regulated by the changes in a protein degradation or production. These types of measurements require simultaneous quantification of isotopic distribution and protein abundance. Also, currently used regression analysis for calculation of a rate constant(s) is based on a single compartmental model that relies on a steady-state assumption. However, amino acids and protein levels are in a non-steady state during growth, aging, and diseases [92]. The nonsteady-state calculations of protein turnover necessitate kinetic models, including data on both protein abundance and relative isotopomer distribution. The future bioinformatics tools based on multi-compartmental kinetic analysis and the quantification of absolute protein production rate in nonsteady-state condition would greatly advance proteome dynamics studies. In addition, there is currently a gap between dynamic proteomics and pathway analysis. Although several software are available for the functional analysis of data based on static proteomics data, currently there are no bioinformatics tools for system biology flux analysis using the proteome dynamics data.

Finally, clinical application of the heavy water method would necessitate fully automated data analysis. So far, existing software solutions are unconnected applications that require multiple format conversion for the input and analysis. Improvement in software cross talk between raw data inputs and data analysis applications would integrate data analysis pipelines with data acquisition and search engines. This would require software engineering development that could transform the existing algorithms to robust user-friendly software packages.

In addition, to the technical limitations highlighted above, the heavy water-based metabolic labeling approach is applicable to analysis of dynamics of proteins with a half-life of greater than ~2 h. This is because it takes approximately 1 h to reach the steady-state enrichment in the amino acid pool, thus it cannot be used for rapidly secreted fast turnover peptides. On the other hand, it is ideally suited to assess proteins that have a more constant rate of secretion and relative stable plasma concentrations, and a half-life of >2 h. It is also not appropriate in short-term experiments (less than 1 week) to measure proteins in plasma that are slowly

http://dx.doi.org/10.5772/61776

synthesized constituents of cells, such as troponin or creatine kinase, released in response to tissue injury or necrosis.

Thus, routine and widespread utilization of ^2H_2O as a diagnostic tool in patient care requires future advancement in several areas. As we discussed above, robust study designs complemented with facile sample preparation, multiplexed analysis, and user-friendly software package allowing high-throughput data processing and interpretation are required. As a universal tracer, heavy water could be used to measure other metabolic fluxes along with proteome dynamics. Thus, as a comprehensive diagnostic tool, the heavy water method could revolutionize personalized medicine, provided there are certain future technological advancements in this field.

Acknowledgements

We thank Dr. Vernon E. Anderson for his insight and efforts in developing early stages of our proteome dynamic studies. This work was funded by National Institutes of Health Grants 5R21RR025346 (SP, TK), R21 HL-114407 (TK), NLBIHHSN268201000037C (RGS), 1RO1GM112044-01A1 (RGS, TK), American Heart Association 131RG14700011, and 15GRNNT25500004 (TK), and was supported in part by the National Institutes of Health, Clinical Translational Science Collaborative of Cleveland UL1TR000439 (TK).

The Thermo Orbitrap Elite mass spectrometer used in this study was purchased using NIH shared instrument grant 1S10RR031537-01 (BW).

Author details

T. Kasumov[1,2*], B. Willard[3], L. Li[3], R.G. Sadygov[4] and S. Previs[5]

*Address all correspondence to: kasumov@neomed.edu or tkasumov@hotmail.com

1 Department of Gastroenterology and Hepatology, Cleveland Clinic, Cleveland, OH, USA

2 Department of Pharmaceutical Sciences, Northeast Ohio Medical University, Rootstown, OH, USA

3 Department of Research Core Services, Cleveland Clinic, Cleveland, OH, USA

4 Department of Biochemistry and Molecular Biology, The University of Texas Medical Branch, Galveston, TX, USA

5 Pharmacokinetics, Pharmacodynamics and Drug Metabolism, Merck NJ, USA

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[93] This paper is dedicated to the memory of Dr. William C. Stanley.

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