Plasma Proteome Dynamics: Analysis of Lipoproteins and Acute Phase Response Proteins with $^{2}$H$_2$O Metabolic Labeling

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Understanding the pathologies related to the regulation of protein metabolism requires methods for studying the kinetics of individual proteins. We developed a $^{2}$H$_2$O metabolic labeling technique and software for protein kinetic studies in free living organisms. This approach for proteome dynamic studies requires the measurement of total body water enrichments by GC-MS, isotopic distribution of the tryptic peptide by LC-MS/MS, and estimation of the asymptotical number of deuterium incorporated into a peptide by software. We applied this technique to measure the synthesis rates of several plasma lipoproteins and acute phase response proteins in rats. Samples were collected at different time points, and proteins were separated by a gradient gel electrophoresis. $^{2}$H labeling of tryptic peptides was analyzed by ion trap tandem mass spectrometry (LTQ MS/MS) for measurement of the fractional synthesis rates of plasma proteins. The high sensitivity of LTQ MS in zoom scan mode in combination with $^{2}$H label amplification in proteolytic peptides allows detection of the changes in plasma protein synthesis related to animal nutritional status. Our results demonstrate that fasting has divergent effects on the rate of synthesis of plasma proteins, increasing synthesis of ApoB 100 but decreasing formation of albumin and fibrinogen. We conclude that this technique can effectively measure the synthesis of plasma proteins and can be used to study the regulation of protein homeostasis under physiological and pathological conditions. Molecular & Cellular Proteomics 11: 10.1074/mcp.M111.014209, 1–16, 2012.

Recent advances in static measurements of genomics and proteomics have greatly enhanced our understanding of genome-proteome interactions. Although static gene expression can be reflected in protein levels (1), it is now well recognized that mRNA and protein levels are poorly associated (2), and there is a clear need for methods that can provide information on the rate of synthesis of individual protein products (3). Protein turnover studies have traditionally relied on precursor-product relationships and involved the administration of isotopically labeled amino acid (4). In addition to the inconvenience of long term administration of amino acid tracers and difficulties in determining the true precursor labeling, these methods are hampered by low isotopic labeling of protein products and difficulties with data interpretation related to determining the precursor-product labeling ratio. Classical protein turnover studies were also often limited to investigating of the kinetics of an individual or mixed protein pool in whole body or specific organs (5). Recent advances in protein sequencing allowed the assessment of the turnover rates of multiple individual proteins simultaneously (6). Stable isotope labeling by amino acids in cell culture (SILAC), originally developed for quantitative analysis of proteins (7), is consequently applied in pulse chase labeling experiments for steady state protein turnover measurements (8). Recently the global metabolic labeling of the entire proteome was achieved using $^{15}$N-labeled medium and diet for cell culture and animal studies, respectively (9, 10). Although these techniques allow estimation of global proteome dynamics without the requirement of precursor isotope abundance, these approaches require a long term time course experiment for exponential fitting. In addition, these techniques are not suited for human studies because they require the consumption of a fully $^{15}$N-labeled diet.

Recently we and others developed the $^{2}$H$_2$O metabolic labeling technique to measure protein turnover in free living animals (11–15). In rats that have been given intraperitoneal injection of $^{2}$H$_2$O, $^{2}$H equilibrates with total body water and...
amino acids such as alanine within 10 and 20 min, respectively (16) (Fig. 1). The enzymes of intermediary metabolism transfer 2H from body water to nonessential amino acids, whereas all free amino acids, including essential amino acids, are labeled through transamination (16). Recently we demonstrated that the steady state 2H labeling of most intracellular amino acids was achieved within 45 min of an intraperitoneal bolus of 2H2O, suggesting that the transfer of amino acids to the polypeptide chain is the rate-limiting step in protein biosynthesis (12, 17). The incorporation of multiple copies of 2H from 2H2O to nonessential amino acids (alanine, glutamine, glutamate, glycine, serine, etc.) results in amplification of isotopic enrichment in a product and facilitates measurement of their labeling. Previously the use of 2H2O was hampered by the complexity of the isotopomer distribution of 2H-labeled peptide. Recently we developed a mathematical algorithm for the analysis of the mass isotopomer distribution and measurement of protein dynamics using high resolution FT-ICR MS (13). This approach is based on the concept that 2H2O is the tracer precursor and the proteolytic peptide is the product, which accumulates multiple copies of 2H from 2H-labeled amino acids. The predicted number of 2H incorporated into a peptide is calculated by the software, which estimates the isotopomer distribution of a peptide based on the number of exchanged hydrogen atoms and the enrichment of total body water (TBW) and then finds the best fit for the experimentally observed isotope envelope. Although FT-ICR MS is an excellent choice for isotopic ratio measurements, we suspect that the high cost of this instrument precludes its routine application for protein turnover studies. In this study, we tested the utility of a more commonly available ion trap LTQ MS instrument to study proteome dynamics in rat plasma through a 2H metabolic labeling approach, because such instruments are more routinely used in proteomic investigations. We expected that because the LTQ MS in zoom scan mode has high spectral accuracy and precision, it would accurately measure peptide labeling; therefore LTQ MS should enable the measurement of plasma protein synthesis in a few hours of labeling experiment. In this study, we mainly concentrated on several plasma acute phase response proteins and lipoproteins and evaluated the effects of short term fasting.

EXPERIMENTAL PROCEDURES

Materials

HPLC grade solvents for nanospray chromatography and sample preparation were purchased from Fluka (Milwaukee, WI). All other chemicals were from Sigma-Aldrich.
All of the animal procedures were approved by the institutional animal care and use committee at the Cleveland Clinic and were performed in accordance with National Institutes of Health guidelines. Male Sprague-Dawley rats (250 g) were purchased from Charles River Laboratories (Wilmington, MA) and were housed in our animal care facility with a 12:12 h light-dark cycle. The animals had free access to food (20% kcal from protein, 70% kcal from carbohydrate, and 10% kcal from fat; Harlan Teklad) and water.

After a 3-day quarantine period, the animals were housed in separate cages, and their daily food intake was recorded for 1 week. A second group of rats (20 animals) was randomly divided into fed and fasted groups. The rats from the fed group had full access to food, whereas food was removed from the fasted group at 5:00 p.m. the day before 2H2O administration, food was removed from animals in the fasted group (studied in the short term experiment). The samples were collected at different time points (see “Experimental Procedures”). The proteins were separated using a gradient SDS-PAGE and analyzed by ion trap LC-MS/MS.

The next day, the rats were injected with 2H2O (20 μl/g of body weight), rats had free access to drinking water containing 5% 2H2O. The animals used in long term experiments also had full access to food. At 5:00 p.m., the day before 2H2O administration, food was removed from animals in the fasted group (studied in the short term experiment). The samples were collected at different time points (see “Experimental Procedures”). The proteins were separated using a gradient SDS-PAGE and analyzed by ion trap LC-MS/MS.

Animal Studies

After bolus injection of 2H2O (20 μl/g of body weight), rats had free access to drinking water containing 5% 2H2O. The animals used in long term experiments also had full access to food. At 5:00 p.m., the day before 2H2O administration, food was removed from animals in the fasted group (studied in the short term experiment). The samples were collected at different time points (see “Experimental Procedures”). The proteins were separated using a gradient SDS-PAGE and analyzed by ion trap LC-MS/MS.

2H enrichment of TBW was measured using a modification of the acetone exchange method (19). Measurement of the 2H enrichment of water is based on the isotopic exchange between the hydrogens of water and of acetone in alkaline medium (pH 12–13). Because acetone has six exchangeable hydrogen atoms, the 2H enrichment of water could be amplified up to 6-fold in the M1/M0 ratio of acetone, which improves the sensitivity of the assay.

The second group of rats (20 animals) was randomly divided into fed and fasted groups. The rats from the fed group had full access to food, whereas food was removed from the fasted group at 5:00 p.m. the day before the metabolic labeling experiment. The following morning, the rats were injected with 2H2O (20 μl/g of body weight) and then they had free access to drinking water enriched with 2H2O (5%) and food. The animals were euthanized 1, 3, 5, 7, and 10 days after the labeling experiment.

To measure free amino acid labeling in rat liver, 0.15–0.2-g liver samples were homogenized in 1 ml of 6% formic acid (20). After centrifugation, the supernatants were diluted with two volumes of water and loaded on an ion exchange column (AG 50W-X8 resin, hydrogen form). The column was first washed with water (5 ml), and then they had free access to drinking water enriched with 2H2O (5%) and food. The animals used in long term experiments also had full access to food. At 5:00 p.m., the day before 2H2O administration, food was removed from animals in the fasted group (studied in the short term experiment). The samples were collected at different time points (see “Experimental Procedures”). The proteins were separated using a gradient SDS-PAGE and analyzed by ion trap LC-MS/MS.

2H Labeling of Amino Acids

To measure free amino acid labeling in rat liver, 0.15–0.2-g liver samples were homogenized in 1 ml of 6% formic acid (20). After centrifugation, the supernatants were diluted with two volumes of water and loaded on an ion exchange column (AG 50W-X8 resin, hydrogen form). The column was first washed with water (5 ml), and then amino acids were eluted with 4 N ammonium hydroxide (5 ml). The eluent was dried, and the residue was derivatized with 80 μl of bis(trimethylsilyl) trifluoroacetamide + 10% trimethylchlorosilane (Pierce) at 80 °C for 2 h (21). The trimethylsilyl derivatives of amino acids were analyzed using GC-MS (an Agilent 5973N-MSD instrument equipped with an Agilent 6890 GC system). One microliter of the trimethylsilyl derivative was injected into a gas chromatograph equipped with a DB-17MS capillary column (30 m × 0.25 mm × 0.25 μm). GC-MS operation conditions were as follows: injector temperature, 240 °C; transfer line temperature, 310 °C; split ratio, 1/30; oven,
Protein Separation

Proteins from the plasma sample were isolated by 50% isopropanol precipitation. Proteins from the pellet and supernatant fractions were further separated by SDS-PAGE.

Pellet Protein Preparation

Rat plasma samples (50 µl) were diluted with 700 µl of 0.15 M NaCl solution. Then 750 µl of isopropanol was added, and the mixture was incubated at 4 °C for 1 h. After centrifugation for 10 min, the supernatant was carefully removed without disturbing the pellet residue. The supernatant was saved for the analysis of soluble proteins. The pellets were washed twice with cold 50% isopropanol and air-dried. The supernatant was removed without disturbing the pellet residue. After centrifugation for 10 min, the supernatant was carefully removed without disturbing the pellet residue. The supernatant was saved for the analysis of soluble proteins. The pellets were washed twice with cold 50% isopropanol and air-dried. The supernatant was removed without disturbing the pellet residue.

Soluble Protein Preparation

The supernatant saved from the 50% isopropanol precipitation step was evaporated down to ~500 µl, and then the proteins were precipitated with 500 µl of 10% Trichloroacetic acid. The residue was centrifuged and washed twice with 100 µl of acetone at ~20 °C. The air-dried residue was dissolved in 40 µl of 1× Laemmli sample buffer (Bio-Rad) (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromphenol blue, and 0.125 m Tris-HCl) and 0.7 M dithiothreitol (DTT) was added. The solution was incubated in a boiling water bath for 5 min. The samples were separated by 5–20% PAGE for 1 h at 200 V.

In-gel Digestion

Protein gel bands were excised from SDS-PAGE gels, and the bands were cut from the gel as close as possible to minimize excess polyacrylamide, divided into a number of smaller pieces (~1 mm × 1 mm), washed, and destained in 50% ethanol, 5% acetic acid, and reduced with DTT and carbamidomethylated with iodoacetamide at room temperature. The proteins in the gel bands were digested with excess amount of Promega sequencing grade trypsin (20 µl of 20 ng/µl trypsin in 50 mU ammonium bicarbonate) overnight at room temperature. Tryptic peptides were extracted from the polyacrylamide in two aliquots of 30 µl of 50% acetonitrile with 5% formic acid. These extracts were combined, evaporated, and reconstituted in 30 µl of 1% acetic acid. Five and 10 µl of sample were injected for the LC-LTQ-FT-ICR-MS and LC-LTQ-MS/MS analyses, respectively.

Proteomics-based Analysis by Nanospray LC-MS/MS

Proteomics-based Analysis by Nanospray LC-MS/MS—Chromatographic separation of the protein digest was performed by an Ultimate 3000 nano HPLC (Dionex, Germering, Germany) with a trapping desalting precolumn (C18, PepMap100, 300 µm × 5 mm, 5-µm particle size, 100 Å; Dionex) followed by a reverse phase column (C18, 75 µm × 150 mm, 10 µm; Dionex), using a mobile phase A (0.1% formic acid in water) and B (80% acetonitrile, 0.04% formic acid in water) gradient.

Tandem mass spectra were recorded on a Finnigan LTQ FT ULTRA hybrid mass spectrometer (Thermo Electron Corp., Bremen, Germany) equipped with a 7 T superconducting electromagnet and a Packed-Tip nanospray ionization probe (Thermo Electron Corp., Thermo Fisher Scientific) operated in a positive ion mode. The peptides were infused at a flow rate of 300 nl/min via the silica noncoated PicoTip emitter (FS360-20-10-C12; New Objective Inc., Woburn, MA) at a voltage of 2.4 kV. The inlet capillary temperature was maintained at 200 °C.

The isotopic detection was performed in the ICR cell. The peptides ions were monitored in the selected ion monitoring mode (mass interval is 10 Th) at a resolution of 12,500. In parallel with ICR measurements, the MS/MS spectra of selected peptides were produced in LTQ for the confirmation of the peptide sequence.

LC-LTQ-MS/MS analysis—Chromatographic separation of the protein digest was performed by an Eksigent NanoLC-1Dplus HPLC (Eksigent Technologies Inc.) with a reverse phase custom made nanocolumn (C18, 75 µm × 100 mm, 10 µm; New Objective) using a mobile phase A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile). A 71-min linear gradient started with 100% of phase A; after 5 min of desalting, the phase B was increased to 13% in 1 min and then linearly to 26% in 40 min. Subsequently the column was washed for 10 min with 100% of mobile phase B and equilibrated for 15 min with 100% of A. Tandem mass spectra were recorded on a Finnigan LTQ (Thermo Electron Corp., Bremen, Germany) operated in a positive ion mode. The peptides were infused at a flow rate of 300 nl/min via the silica noncoated PicoTip emitter (FS360-75-15-N-512; New Objective Inc., Woburn, MA) at a voltage of 2.5 kV. The inlet capillary temperature was maintained at 200 °C. Each full MS scan spectrum was followed by five MS/MS spectra. The dynamic exclusion option was enabled after three repeat acquisitions within a 15-s duration. Only singly charged MS/MS product ions with good precursor abundance (10⁵–10⁶ intensity) were selected for an accurate quantification of label incorporation. Additional criteria for peptide selection included: (i) unique to the protein (confirmed by Blast search); (ii) no isobaric overlapping (mass isotope distribution differs within 1–1.5% from the theoretical); (iii) a peptide with a Mascot score of higher than 50; (iv) good chromatographic properties (elution time, and chromatographic peak shape); (v) high intensity (10⁴–10⁶ in our instrument) of a peptide and its fragment ions; and (vi) high content of alanine, glutamine, glutamate, glycine, and arginine in the peptide sequence.

Once the peptides for quantification were selected from the data-dependent survey analysis, the CID of doubly charged peptides was performed in a selected reaction monitoring (SRM) mode with zoom scan of an abundant singly charged product ion. The doubly charged parent ions m/z (Q) were monitored with the isolation width of 6 Th (from Q − 0.5 to Q + 5.5), whereas the zoom scan of the fragment ions m/z (D) were recorded within an isolation window of 10 Th (from D − 2 to D + 8). Isotope incorporation was assessed based on the mass isotopomer² distribution analysis of a product ion from the zoom scans. Only averaged peaks satisfying Gaussian peak shape without interfering isobaric peaks were used for the analysis. The quantification was performed by integrating each isotopomer of a given chromatographic peak within a defined mass range (Mi ≥ 0.5 Th). The precision (i.e., intra- and interassay reproducibility) of ²H labeling measurements was determined by multiple analyses of a plasma sample. Intra-assay variability was determined by analyzing one sample five times. Interassay variability was established by proc-
Database Searching

For the identification of proteins, the MS data were analyzed using all CID spectra collected in the experiment. The peak lists were extracted using Thermo Scientific Electron Xcalibur v2.0 subroutine extract_msn.exe and were generated for all peaks with at least 15 product ions in the MS/MS spectra. The peak lists were searched using the program Mascot version 2.3 (Matrix Science, London) against the National Center for Biotechnology Information Rattus norvegicus fasta database (ftp://ftp.ncbi.nih.gov/refseq/) released on July 17, 2006 containing 36,359 entries. The search was performed using carbamidomethyl as a fixed modification of cysteine, using oxidation as an optional modification of methionine, and allowing one missed cleavage. The mass tolerances for the precursor and product ions are 3.0 and 1.5 Th, respectively. A score of >30 was considered as significant. The interpretation process was aided by additional searches using the programs Sequest, which is bundled into BioWorks Browser version 3.1 (Thermo Electron Co.) and Blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi) as needed.

Calculations

The 2H labeling of an amino acid and a peptide were calculated by normalizing the M1 isotopomer against the M0 isotopomer. Although our approach does not require the background correction for natural enrichment, the isotopic excess caused by 2H incorporation was calculated via subtraction of the base-line ratio calculated for a control rat.

Because the synthesis of a protein tends to be independent of protein concentration and follows zero order kinetics, the fractional synthesis rate (FSR) in the long term experiment was determined using a one compartment model by fitting a time course of total labeling of a peptide (E_p) to the following exponential rise curve equation.

\[ E_{\text{peptide}}(t) = E_{\text{ss}} \cdot (1 - e^{-kt}) \]  

(Eq. 1)

This equation allows determination of asymptotical steady state labeling (E_ss), the rate constant (k), and the half-life (t_half = ln2/k) of a protein. The calculations of these parameters do not require the measurement of precursor labeling; however, it necessitates a long term experiment with sufficient data points for the exponential fitting. Note that at steady state, the turnover rate constant represents both the FSR and the fractional catabolic rate.

By definition, the time domain derivative of Equation 1 represents the slope of the tangent line at the initial semi-linear part of E_peptide(t) function: Slope = \( \frac{(E_{\text{peptide}}(t))'}{E_{\text{ss}}} \cdot k \cdot E_{\text{ss}} \cdot (1 - e^{-kt}) \) and by the arrangement \( k = \text{slope}/E_{\text{ss}} \cdot e^{-kt} \).

Therefore, when t \( \rightarrow 0 \), the turnover rate constant (k) represents the FSR in the short term experiment.

\[ \text{FSR} = k = \text{slope}/E_{\text{ss}} \]  

(Eq. 2)

Because the plateau labeling (E_ss) depends on TBW labeling and the asymptotical number of exchanged H atoms (N) (13),

\[ E_{\text{ss}} = E_{\text{water}} \cdot N \]  

(Eq. 3)

Thus,

\[ \text{FSR} = \text{slope of product labeling}/(E_{\text{water}} \cdot N) \]  

(Eq. 4)

Where the slope is the rate of increase in 2H labeling of peptide, and E_water is the steady state enrichment of the TBW. The ability to estimate FSR in a short term experiment requires measurements of peptide labeling by LC-MS/MS, water labeling by GC-MS, and the calculation of the asymptotic number of deuterium incorporated into the peptide (N) using a mathematical algorithm (see below). Consequently, the formula for calculating the FSR in a short term experiment is based on the precursor-product relationship assuming that 2H2O is the precursor and a proteolytic peptide is the product. In this study, we demonstrated that, after administration of 2H2O, 2H equilibrium between total body water and intracellular amino acids is very rapid, and steady state enrichment values were obtained within 60 min (see “Results”). This validates the assumption that 2H2O is a true 2H label precursor for protein synthesis.

Although this approach requires a determination of the TBW labeling, it substantially shortens the duration of a 2H2O metabolic labeling experiment. A similar approach has been widely used to calculate of FSR for cholesterol and fatty acids (22, 23). N is a constant number for a specific lipid (as we assumed here for a peptide). Once N is determined from a long term experiment, it could be used for calculation of the FSR in any short term experiment. In contrast to the long term experiment requiring a time span equivalent to four to five half-lives of the specific protein, the FSR in the short term experiment can be calculated based on the early hours of the labeling experiment. In addition, because 2H2O readily equilibrates with TBW in all cellular compartments, the precursor labeling can be easily and noninvasively measured in plasma or urine.

Calculation of Asymptotical Number of 2H in Carbon-Hydrogen Sites

The number of exchanged hydrogen atoms in each analyzed peptide was calculated using software previously described (13). Briefly, this software generates the mass isotopomer distribution of a peptide based on plasma 2H2O labeling and the number of incorporated 2H atoms and compares that with experimentally measured plateau labeling of a peptide. The mean sum of square errors is used to fit the predicted data to the experimental results. Thus, for a specific peptide, the best fit of N is determined based on the minimum error between the theoretical isotopic distribution simulated by the program and the experimentally measured isotope distribution at steady state. Because the asymptotical number of 2H in carbon-hydrogen sites for a peptide is calculated based on the plateau labeling of the peptide, this requires the steady state 2H2O-labeling experiment which typically covers four to five half-lives of a protein. During this time period, almost the entire pool of a protein is completely turned over, indicating that the number of incorporated deuterium atoms reflects the maximum possible incorporation.

Data Presentation and Statistical Analysis

The data are shown as the excess labeling in a given rat experiment, and each symbol in a figure corresponds to the value calculated based on one rat experiment. To analyze data from long term experiments, a time course of the relative 2H excess curve was constructed for several (n = 3–6) peptides of each protein. Aggregation of the relative 2H excess curve of a peptide into protein curves involves the normalization of peptide labeling at each time point to the plateau labeling, averaging and fitting into the exponential rise curve. The error bars in these curves represent standard deviations based on all normalized peptide labeling.

Linear regression analysis was used to assess the relationship between peptide labeling and time; this was done for each nutritional status (short term experiment) separately. The statistical significance of differences between the profiles of 2H enrichments (i.e., different slopes) of a selected peptide in fed and fasted experiments were tested using a model containing time, nutritional status, and the
interaction term between them as the independent variables for each peptide. A \( p < 0.05 \) was considered statistically significant. All analyses were performed using SAS (version 9.2; The SAS Institute, Cary, NC) and R (version 2.13.1; The R Foundation for Statistical Computing, Vienna, Austria).

### RESULTS

**Animal Experiments**

It was previously demonstrated that the chronic intake of \( \text{D}_{2}	ext{H}_{2}O \) at 20% enrichment in drinking water (which results in \( \sim10\% \) of the body water enrichment with \( \text{D}_{2}	ext{H}_{2}O \) does not affect growth, food intake, behavior, activity, or fertility in mice (24). In this study we used a similar protocol, but with a 4-fold lower \( \text{D}_{2}	ext{H}_{2}O \) enrichment in drinking water. All of the animals were examined daily for signs of health problems, food intake, and body mass. No adverse effects on growth or food consumption were observed during the 10 days of \( \text{D}_{2}	ext{H}_{2}O \) intake. The steady state labeling of TBW (2.65 \( \pm \) 0.12) was achieved within 30 min.

To determine the extent to which the precursor proteogenic amino acid pool is labeled, after an intraperitoneal injection of \( \text{D}_{2}	ext{H}_{2}O \), \( \text{D}^{3} \) labeling of hepatic free amino acids at various time points was assessed by GC-MS (Table I). After 30 min of \( \text{D}_{2}	ext{H}_{2}O \) administration, different amounts of \( \text{D}^{3} \) label incorporation were found in all the measured 18 amino acids. Furthermore, the labeling of all these amino acids reached steady state within 1 h of administrating the \( \text{D}_{2}	ext{H}_{2}O \) bolus. Consistent with previous reports (12, 15, 17), alanine, glutamate, glutamine, glycine, and serine were extensively labeled, reflecting their central role in intermediary metabolism. Small, in some cases negligible, amounts of \( \text{D}^{3} \) label were found in most essential amino acids. Although essential amino acids cannot be synthesized \textit{de novo}, most of them can be completely substituted for in the diet by their \( \alpha \)-keto derivatives. This suggests fast deamination followed by reamination of these amino acids, which theoretically should result in incorporation of one \( \text{D}^{3} \) from TBW. In addition, methionine may incorporate some \( \text{D}^{3} \) through transmethylation of homocysteine. As we found previously (12), the measured \( \text{D}^{3} \) labeling of all amino acids is lower than the theoretically expected maximum number of exchangeable carbon bound H atoms. This incomplete labeling of amino acids may reflect unequal equilibration on different positions of amino acids with TBW.

The very rapid labeling of proteogenic amino acid in rat liver demonstrates their short half-life and excludes the possibility to calculate amino acids turnover rates from their \( \text{D}^{3} \) labeling. However, labeling of nonexchangeable hydrogen atoms in free amino acids within 1 h of \( \text{D}_{2}	ext{H}_{2}O \) administration ensures steady state precursor labeling, a major assumption in a tracer-based kinetic study. Although the immediate precursor pools for protein synthesis are the tRNA-bound amino acids, these amino acid derivatives are in low abundance, and it is difficult to measure them accurately. Therefore, cellular free amino acids are commonly used surrogates of the true precursor pool. While this manuscript was in preparation, a new detailed study on time course equilibration of \( \text{D}^{3} \) labeling between body water and free amino acids was published by

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**Table I**

| # | Derivative | Retention time (min) | M1/M0 | Excess \( \text{D}^{3} \) labeling |
|---|-----------|---------------------|-------|------------------------|
| 1 | Alanine   | di-TMS              | 4.96  | 117.2/116.2            |
| 2 | Glycine   | di-TMS              | 5.58  | 103.2/102.2            |
| 3 | Valine    | di-TMS              | 6.71  | 219.2/218.2            |
| 4 | Leucine   | di-TMS              | 7.68  | 159.2/158.2            |
| 5 | Isoleucine| di-TMS              | 8.09  | 159.2/158.2            |
| 6 | Serine    | tri-TMS             | 9.27  | 205.2/204.2            |
| 7 | Threonine | tri-TMS             | 9.50  | 292.1/291.2            |
| 8 | Aspartate | tri-TMS             | 11.78 | 233.2/232.2            |
| 9 | Asparagine| tri-TMS             | 12.78 | 189.2/188.2            |
|10 | Methionine| di-TMS              | 12.96 | 177.2/176.2            |
|11 | Cysteine  | tri-TMS             | 13.02 | 295.2/294.2            |
|12 | Glutamate | tri-TMS             | 13.9  | 247.2/246.2            |
|13 | Phenylalanine| di-TMS | 14.73 | 193.2/192.2            |
|14 | Proline   | di-TMS              | 15.05 | 143.2/142.2            |
|15 | Glutamine | tri-TMS             | 16.76 | 246.2/245.2            |
|16 | Lysine    | tetra-TMS           | 16.82 | 318.2/317.2            |
|17 | Tyrosine  | tri-TMS             | 18.42 | 281.2/280.2            |
|18 | Histidine | tri-TMS             | 19.28 | 255.2/254.23           |
|19 | Tryptophan| tri-TMS             | 22.65 | 304.2/303.2            |

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Previs and co-workers (20) at Merck. Similar to our results, their data demonstrated that $^2$H from body water equilibrated with the carbon-bound hydrogen of amino acids within the time frame of the experiment.

**Protein Isolation and Identification**

Blood samples from rats with different durations of metabolic labeling were collected and analyzed for plasma protein kinetics. Our analysis concentrated on several plasma acute phase response proteins and lipoproteins. Gradient ultracentrifugation is the main method for isolation of different lipoprotein particles. To increase the yield of isolation of these lipoproteins from small volume plasma samples (25 μl) without prior ultracentrifugation, we used an isopropanol precipitation (50% isopropanol solution in water) method. This method concentrates ApoB 100, fibrinogen, and ApoH in the pellet fraction, whereas ceruloplasmin, transferrin, ApoA IV, haptoglobin, ApoE, C-reactive protein, and ApoA I were isolated in a supernatant. Albumin, the most abundant plasma protein, was present in both pellet and supernatant fractions. Each fraction was purified by SDS-PAGE. Trypsin-digested samples from control rats (sham, H2O-treated) were analyzed on an LTQ system using data-dependent survey analysis to identify specific peptides that would be suitable for isotopic distribution analysis in subsequent SRM and selected ion monitoring experiments. To test whether there is any back-exchange of hydrogen atoms during one-dimensional PAGE, the labeling of albumin was analyzed after undergoing in solution and in gel digestions. Both methods yield similar results for the measured labeling of albumin peptides, indicating that electrophoresis does not result in any measurable back-exchange (supplemental Table S1). Table II displays the list of all of the analyzed plasma proteins with characteristic mass, matched peptide number, score, percentage of coverage, and their respective biological role.

### Table II

**Analyzed proteins in rat plasma**

| Protein name | Accession number | Molecular mass (kDa) | Mascot searching result | Biological significance |
|--------------|------------------|----------------------|-------------------------|------------------------|
| Albumin      | gi 19705431      | 70.7                 | 56                      | 63                     | 17626 | The major plasma protein in mammals involved in maintenance of osmotic pressure and transport of hydrophobic steroid hormones and fatty acids |
| ApoA I       | gi 6978515       | 30.1                 | 27                      | 65                     | 3395  | The major protein component of high density lipoprotein in plasma, promotes cholesterol efflux from tissues to the liver for excretion |
| ApoA IV      | gi 8392909       | 44.4                 | 15                      | 44                     | 1343  | The primary protein of very low density lipoprotein and low density lipoprotein responsible for carrying cholesterol to tissues |
| ApoB 100     | gi 61098031      | 537.7                | 38                      | 13                     | 1054  | The primary protein of very low density lipoprotein and low density lipoprotein responsible for carrying cholesterol to tissues |
| ApoE         | gi 20301954      | 36.1                 | 12                      | 29                     | 2054  | Essential for the normal catabolism of triglyceride-rich lipoprotein constituents |
| ApoH         | gi 57528174      | 39.7                 | 14                      | 44                     | 4221  | It binds to cardiolipin and completely inhibits serotonin release by the platelets and prevents subsequent ADP-induced aggregation |
| C-reactive Protein Ceruloplasmin | gi 8393197 gi 6978695 | 25.7 gi 121.6 | 9 gi 36 | 41 gi 29 | 7663 gi 5389 | An inflammatory biomarker |
| Haptoglobin  | gi 60097941      | 39.1                 | 31                      | 54                     | 4683  | It binds free hemoglobin released from erythrocytes with high affinity and thereby inhibits its oxidative activity |
| Transferrin  | gi 61556986      | 78.5                 | 74                      | 61                     | 20506 | Iron-binding blood plasma glycoprotein that controls the level of free iron in biological fluids |
| Fibrinogen β polypeptide | gi 29789106 | 54.9 | 54 | 66 | 9322 | As the precursor of fibrin, can form bridges between platelets; may be elevated in any form of inflammation |

Mass Isotopomer Distribution Analysis of Peptides from Selected Proteins

Spectral Accuracy and Precision of the Mass Isotopomer Analysis by LTQ MS—The spectral accuracy of a mass spectrometer and the reproducibility of isotopic ratio measurements are critical for mass isotopomer distribution analysis. Previously we used high resolution MALDI-TOF or nano-spray liquid chromatography coupled hybrid LTQ FT-ICR instru-
Plasma Proteome Dynamics

Spectral accuracy of mass isotopomer analysis in LTQ MS and FT-ICR

| Protein name   | Peptide sequence | Experimental isotopic distribution (%) | Theoretical isotopic distribution (%) |
|----------------|------------------|----------------------------------------|---------------------------------------|
|                |                  | M1/M0       | M2/M0       | M3/M0       | M4/M0       | M1/M0       | M2/M0       | M3/M0       | M4/M0       |
| ApoA_1         | ADAALR           | 26.55       | 6.01        | 0.80        | 0.13        | 28.48       | 5.55        | 0.81        | 0.10        |
| ApoA_IV        | PLVEDVQSK        | 53.32       | 15.50       | 3.47        | 0.76        | 55.04       | 18.13       | 4.42        | 0.88        |
| ApoB 100 (LTQ) | PAEQTSNQK        | 58.79       | 20.23       | 4.93        | 1.73        | 56.41       | 18.89       | 4.67        | 0.91        |
| ApoB 100 (FT-ICR)| GFEPTLEALFGK     | 73.84       | 30.33       | 2.50        | 0.30        | 76.27       | 32.37       | 9.30        | 2.41        |
| ApoE           | VGQAR            | 32.94       | 8.62        | 0.30        | 3.48        | 33.06       | 7.13        | 1.66        | 0.16        |
| ApoH           | PPRPITK          | 45.17       | 11.80       | 2.15        | 0.38        | 45.57       | 11.78       | 2.21        | 0.33        |
| C-reactive Protein | SIFSYYATK       | 50.42       | 15.13       | 3.09        | 0.55        | 52.92       | 16.38       | 3.74        | 0.68        |
| Ceruloplasmin  | AFFQVR           | 44.51       | 13.02       | 2.40        | 0.55        | 46.22       | 12.08       | 2.29        | 0.35        |
| Haptoglobin    | PTLTLVYQK        | 57.40       | 18.67       | 4.24        | 0.54        | 57.96       | 19.15       | 4.61        | 0.89        |
| Transferin     | SQGCPAGYK        | 52.18       | 20.23       | 6.09        | 2.47        | 51.45       | 20.26       | 5.85        | 1.37        |
| Fibrinogen β polypeptide | ATNEDTKK    | 46.90       | 14.71       |              |             | 45.90       | 13.57       | 3.00        | 0.55        |

For the kinetic analysis of plasma proteins, we selected four or five peptides from each protein. To improve sensitivity and the isotopic ratio accuracy, the MS analysis was divided into segments for monitoring selected peptides in a single chromatographic acquisition. One or two SRM experiments were performed in each time segment. The SRM experiment is accomplished by specifying the mass of the selected precursor peptide for MS/MS fragmentation and then specifically monitoring for a single charged fragment ion. To obtain the signal of an isotopomer with Gaussian peak shape without interfering peaks, the zoom scans of the most abundant singly charged product ions were performed in a carefully isolated mass range of precursor ion in Y mode. Fig. 4 represents the data-dependent full scan spectra and time-dependent isotopic mass distribution of selected peptides of several plasma proteins. Mass isotopomer distribution analysis demonstrates that the changes in peptides isotopic ratios depend on their protein of origin and the peptide sequence. Consistent with the known half-lives of proteins, the increase in labeling of ApoA I-derived peptides is much slower than peptides derived from ApoA IV, ApoB 100, ApoE, and ApoH. As expected, the ApoE-derived GPAEETR peptide, which is rich with nonessential amino acids (e.g. glycine, alanine, and two glutamate residues), incorporates more ²H than other peptides (Fig. 4).

Before using the LTQ instrument for large scale protein turnover studies, we performed comparative analysis on the kinetics of albumin, ApoB 100, and transferrin by the FT-ICR and LTQ instruments (Fig. 5). The peptide labeling data were fit to a single compartmental model, thus yielding the asymptotic labeling of each peptide and the steady state rate constants of each protein. Although the higher resolution, sensitivity, and mass accuracy of the FT-ICR MS is expected to yield more accurate data without interfering signals, the zoom scan mode in LTQ MS enables reliable measurements of the isotopic ratios. Despite the fact that different peptides were selected for the analysis, similar rate constant values were obtained using these two instruments (Fig. 5).

The data in Fig. 6 show the time course of M1 labeling normalized to M0 of each proteolytic peptide for a different protein from the long term experiment. Equilibrium of the labeling of body water and free amino acids within 1 h enables the FSR measurements of proteins with a half-life as short as ~4 h, i.e., ApoB 100 and ApoA IV. High sensitivity and accuracy of isotopic measurements allows the study of the kinetics of several plasma proteins with a wide range of half-lives during the 7-day labeling experiment. The data in Table IV show the amino acid composition, mass to charge ratio of a proteolytic peptide, asymptotic number of ²H atoms, and the kinetic parameters of each protein (the turnover rate constant...
and half-life) calculated, based on exponential labeling rise curves of analyzed peptides. As expected, peptides originating from the same protein displayed similar turnover rate constants. The calculated half-lives for the analyzed proteins are similar to previously reported values using traditional isotopic amino acid tracers (27–32) (Table IV). Note that the calculated asymptotic number of $^2$H atoms in carbon-hydrogen sites for each analyzed peptide (fourth column) is needed to calculate the FSR of a protein in a short term experiment when labeling of the analyzed peptide increases linearly.

Effect of Fasting on Plasma Protein Turnover in Rats—In contrast to the long term labeling experiment, the short term labeling study results in low labeling of analyzed peptides, which constrains the accuracy of isotopic ratio measurements. To test the utility of the ion trap instrument for providing an accurate measurement of protein kinetics in the short term experiment, ApoB 100, transferrin, and albumin kinetics were analyzed in rats with an 8-h labeling experiment by both FT-ICR and LTQ instruments. As illustrated in Fig. 7, that there is a $\sim$3-h time lag between $^2$H$_2$O administration and effective proteolytic peptide labeling in the short term experiment as observed in both instruments. Both instruments gave similar results for all three analyzed proteins (Table V). Next we assessed the effect of fasting on the FSR of plasma proteins using LTQ MS. The data presented in Fig. 8 show that fasting has a differential effect on the synthesis of selected plasma proteins. Table VI displays the calculated FSR of plasma proteins in fed and fasted rats during the short term labeling experiment. The bolus administration of heavy water followed by the animals free access to drinking water for 7–8 h allows an accurate measurement of protein synthesis in both fed and fasted animals. Because of the incorporation of multiple cop-
ies of $^2$H into tryptic peptides, the $^2$H enrichment of the peptides is amplified. This allows detection of changes in protein synthesis related to the nutritional status of a rat. Our data demonstrate that the FSR of ApoB 100 was increased ($p < 0.05$), whereas the production of albumin and fibrinogen were reduced after fasting ($p < 0.05$). However, no significant changes were observed in the FSR of ApoA I, ApoA IV, ApoE, ApoH, C-reactive protein, transferrin, or ceruloplasmin.

**DISCUSSION**

We used $^2$H$_2$O metabolic labeling to assess large scale plasma proteome dynamics in vivo. Administration of $^2$H$_2$O in a bolus injection followed by 5% supplementation in the drinking water enabled accurate measurement of the FSR of multiple plasma proteins in less than 8 h. This approach considers $^2$H$_2$O as the labeling precursor and is based on the assumption that $^2$H from $^2$H$_2$O rapidly equilibrates with the carbon-bound hydrogen atoms of proteogenic amino acids before they are incorporated into newly synthesized proteins (12, 17). This key assumption was validated based on the analysis of temporal changes in $^2$H labeling of body water and hepatic proteogenic amino acids. The steady state labeling of nonexchangeable hydrogen atoms in free amino acids within 1 h of $^2$H$_2$O administration demonstrates that the rate-limiting step of $^2$H incorporation into long-lived proteins ($t^{1/2} > ~3$ h) is protein synthesis from amino acids. Protein synthesis is assessed from the rate of $^2$H incorporation into a proteolytic peptide; thus, measurements of precursor and product labeling in plasma samples in combination with a mathematical algorithm enables protein turnover studies in a few hours in
### TABLE IV

Calculated kinetics parameters and maximum number of incorporated deuterium into tryptic plasma protein peptides

| Protein name | Peptide sequence | m/z     | N  | SSE   | Observed turnover rate constant (%/h) | Calculated half-life (h) | Reported half-life | Protein concentration (mg/dl) | Total plasma protein (%) |
|--------------|------------------|---------|----|-------|---------------------------------------|--------------------------|---------------------|-----------------------------|--------------------------|
| ApoA I       | ADA   | 545.34 | 11 | 8E-5 | 4.05 ± 0.47                          | 17.3 ± 2.0               | 10.2 h (48)        | 45 ± 1 (56)                | 0.77                     |
|              | AEEDR | 541.30 | 14 | 1.2E-4 | 5.9E-5 | 2.82 ± 0.74                          | 14.7 ± 2.3               | 8.5 h (48)        | 12.5 ± 1.6 (57)            | 0.21                     |
| ApoA IV      | PLYEDVQS    | 1014.5 | 10 | 3E-5 | 5.9E-5 | 10.02 ± 0.90                          | 17.0 ± 0.6               | 7.0 ± 0.6        | 10 ± 3 (58)                | 0.17                     |
|              | APEQTS     | 907.9  | 14 | 6.9E-4 | 3.8E-5 | 10.02 ± 0.90                          | 17.0 ± 0.6               | 7.0 ± 0.6        | 10 ± 3 (58)                | 0.17                     |
| ApoB 100     | PAVEQTSRN   | 1033.3 | 15 | 9E-5 | 16.60 ± 2.06                          | 4.2 ± 0.5                | 2.1 h (mouse) (49)| 10 ± 3 (58)                | 0.41                     |
|              | GFEXTLEALFGK | 907.9  | 14 | 3E-5 | 3.8E-5 | 10.02 ± 0.90                          | 17.0 ± 0.6               | 7.0 ± 0.6        | 10 ± 3 (58)                | 0.17                     |
| ApoE         | GFVAETR     | 858.3  | 18 | 1.2E-4 | 16.18 ± 1.10                          | 4.3 ± 0.3                | 7 h (50)           | 24 ± 5 (58)                | 0.41                     |
|              | VGNOAR      | 643.3  | 15 | 2E-5 | 13.54 ± 0.99                          | 5.1 ± 0.4                | 8.5 h (50)        | 24 ± 5 (58)                | 0.41                     |
| ApoH         | PDLPFPAVVPLK | 745.5  | 5  | 9E-5 | 8.45 ± 0.54                           | 8.7 ± 0.6                | 8.5 h (50)        | 24 ± 5 (58)                | 0.41                     |
| C-reactive   | SIFSYATK    | 916.5  | 8  | 9E-5 | 4.32 ± 0.32                           | 16.1 ± 1.2               | 19 h (51)        | <30 (60)                   | <0.51                    |
|              | SPNVNLR     | 985.5  | 7  | 1E-5 | 4.20 ± 0.33                           | 16.6 ± 1.3               | 19 h (51)        | <30 (60)                   | <0.51                    |
| Ceruloplasmin | APFQVR      | 767.4  | 12 | 2E-5 | 4.10 ± 0.67                           | 17.4 ± 2.8               | 12 h (52)        | 35 ± 61                    | 0.60                     |
|              | QGER         | 489.3  | 12 | 4E-3 | 4.06 ± 0.37                           | 17.2 ± 1.6               | 19 h (51)        | <30 (60)                   | <0.51                    |
| Haptoglobin  | PTLTLYVGK   | 991.6  | 6  | 6E-5 | 14.78 ± 1.68                          | 4.8 ± 0.5                | 3.5 days (human) (53)| 50 ± 62                    | 0.85                     |
|              | DWQESTMAK   | 1123.5 | 17 | 6E-5 | 15.00 ± 1.44                          | 4.7 ± 0.4                | 12 h (52)        | 35 ± 61                    | 0.60                     |
| Transferrin  | SQGCA   | 967.4  | 18 | 1.5E-4 | 2.13 ± 0.20                          | 32.8 ± 3.1               | 33 h (54)        | 280–310 (63)              | 5.04                     |
|              | TGAFQCLVEK  | 1152.5 | 19 | 2E-5 | 2.30 ± 0.28                           | 30.3 ± 2.2               | 23.6 h (55)       | 246 ± 16 (64)              | 4.21                     |
| Fibrinogen β | GQFTQ    | 1151.4 | 26 | 9E-5 | 9.35 ± 2.67                           | 8.1 ± 2.3                | 12 h (52)        | 35 ± 61                    | 0.60                     |
| polypeptide  | ATNEDT     | 906.3  | 11 | 9E-5 | 9.31 ± 2.49                           | 8.0 ± 2.1                | 12 h (52)        | 35 ± 61                    | 0.60                     |

*a* Total protein in rat plasma is ~5850 mg/dl according to the cited literature (65, 66).

*b* Peptide was analyzed by an FT-ICR instrument.
cases where the TBW is enriched ~2.5%. Selection of a mass spectrometer for an accurate isotopic ratio measurement is critical for a metabolic labeling study. However, as shown, the FSR measured using the LTQ instrument in this study was identical to those obtained by high resolution FT-ICR MS. Accurate FSR measurement in a relatively low resolution ion trap MS was achieved because the 2H2O metabolic labeling approach results in label amplification. Furthermore, zoom scan data acquisition mode can sufficiently separate and profile singly charged peptide isotopomers.

It is important to note that the in vivo 2H2O metabolic labeling applied in our study differs from the in vitro H/D(2H) exchange technique that is widely used for protein structure analysis (33). In contrast to reversible H/D exchange of labile hydrogen atoms in preexisting proteins, the 2H2O metabolic labeling irreversibly transfers 2H to the carbon backbone of newly synthesized protein. Although both pre-existing and newly synthesized proteins are subject to reversible H/D exchanges of protein amide backbone and hydroxyl-, carboxyl-, and amino-terminal groups, all of these labile 2H atoms are expected to back-exchange during the extensive sample preparation process, and therefore the measured isotopic enrichment represents only irreversible metabolic labeling. We expect that the software we used to simulate the peptide labeling and calculate the asymptotic number of 2H could also be applied to characterizing the H/2H exchange in experiments that probe protein structure.

Another methodological consideration is the possible deuterium isotope effect reflected in the resolution of 2H-labeled and unlabeled peptides caused by chromatographic fractionation (34). Such fractionation depends on mass difference between unlabeled and 2H-labeled molecules, as was previously observed for 3H-coded peptides when 2H8-ICAT reagent was used in comparative proteomics (35). In theory, the chromatographic fractionation of deuterated peptides could complicate the analysis of isotopic distribution (36). However, it is important to note that in contrast to 2H8-ICAT labeling, which results in a difference of 8 units of mass, the 2H2O metabolic labeling technique used in this study mainly affects the M1 isotopomer with a small change in an average mass (maximum ~0.2–0.4 Th at the labeling plateau) of the peptide. Therefore the chromatographic shift in retention time is negligible.

Several methods have recently emerged in the proteomics field for studying protein turnover. SILAC, originally used for relative quantification of proteomes from different physiological states or intervention, was recently adapted for proteome dynamics in cell culture and live animals (8). Because the SILAC method is based on the utilization of a single labeled amino acid (often leucine), it can only determine the turnover rates of peptides with this specific amino acid. In addition, the need to assess the intracellular precursor amino acid labeling may limit application of SILAC to in vivo studies. In contrast, 2H2O as a tracer labels most proteogenic amino acids and thus allows assessment of the kinetics of virtually all proteolytic peptides. Moreover, the 2H2O metabolic labeling incorporates multiple copies of 2H into nonessential amino acids, which results in severalfold higher labeling of amino acids, and even more labeling of peptides consisting of several amino acids, when compared with total body water labeling. Magnification of 2H labeling in a proteolytic peptide enhances the

![Fig. 7. The labeling delay between 2H2O administration and effective labeling of proteolytic peptides observed in LTQ and ICR FT MS instruments.](image)

| Peptide   | LTQ       | FT-ICR    |
|-----------|-----------|-----------|
| FSR (fed) | FSR (fasted) | FSR (fed) | FSR (fasted) |
| Albumin   | PTLVEAAR  | FPNAEFEIK  |
|           | 2.09 ± 0.15 | 2.00 ± 0.11 |
| ApoB 100  | PAEQTSNWK | IAQDVYSTSAATNLK |
|           | 11.62 ± 0.35 | 15.90 ± 0.61 |
| Transferrin| SQGCAPGYK | DFQLFGSPLGK |
|           | 1.43 ± 0.15 | 1.93 ± 0.19 |

**Table V** Utilization of FT-ICR and LTQ mass spectrometers for the measurements of plasma proteins synthesis in a short term experiment with 2H2O metabolic labeling in vivo in rats (comparative analysis)
ment was maintained at over an 8-day labeling experiment in which the TBW enrich-
in mass isotopomer ratio with TBW enrichment of 2.5%.

The turnover rate constants and calculated half-lives of dif-
ferences were observed for the acute phase response proteins haptoglobin and fibrinogen. Albumin, ApoA I, ceruloplasmin, and transferrin involved in fatty acid transport, reverse cholesterol transport, copper metabolism, and iron metabolism, respectively, have the longest half-lives from all the studied plasma proteins. The observed range of half-lives is also in agreement with the N-end rule, which states that the half-life of a protein is determined by the amino-terminal amino acid residues, specific degradation signals called N-degrons (38, 39). ApoB 100, ApoE, and haptoglobin with destabilizing bulky hydrophobic (type 2 N-degrons) and basic amino-ter-

precision and accuracy of the measurements even at lower 2H enrichment of the total body water.

Recently Xiao et al. (37) demonstrated the ability to deter-
mine protein synthesis in vivo using 2H2O and MALDI-TOF MS. Because protein synthesis was estimated using an average mass shift, these investigators were not able to determine the FSR in short term experiments when there was a negligi-
able average mass shift, i.e., protein labeling was assessed over an 8-day labeling experiment in which the TBW enrich-
ment was maintained at ~4%. In contrast, the algorithm used in our study enables an accurate quantification of the changes in mass isotopomer ratio with TBW enrichment of ~2.5% after several hours of labeling experiment.

After the validation of this technique, we used the 2H labeling
approach to measure the turnover rates of several acute phase response proteins and lipoproteins in rat plasma. We observed the expected differences in the FSR for the different proteins (Table IV). As shown, this technique allows measurement of the kinetics of proteins with a wide range of rate constants (~1%/h for albumin and ~16%/h for ApoB 100). The turnover rate constants and calculated half-lives of dif-

The results obtained from the long term experiments were
used to calculate the maximum number of 2H incorporated
into the tryptic peptides; this parameter is needed to estimate
the FSR in short term experiments. For most of the analyzed
proteins, the FSR observed in a long term experiment is
similar to that in a short term experiment. However, a slightly
higher discrepancy between the FSR from the two experi-
ments was observed for proteins with shorter half-lives, i.e.,
ApoB 100 and ApoE. This is because the turnover rate con-
stant overestimates the FSR for proteins that have an FSR
that is greater than 50% during that time period (9). There is a
time delay between 2H2O administration and the effective
onset labeling of proteins as assessed based on all analyzed
tryptic peptides from different proteins. This delay is espe-
cially important in cases where FSR is relatively fast, e.g.
ApoB and ApoE. Such delays are expected and most likely
reflect a lag between hepatic protein synthesis and secre-
tion (40). Secretory proteins are synthesized on polysomes
bound to rough endoplasmic reticulum (ER) and are
cotranslationally transported to the lumen of the ER. Before
secretion, proteins are transported from the ER to the Golgi
apparatus. There is a temporal delay in the transfer from the
ER, which is regulated by the microsomal membrane asso-
ciation of proteins. Albumin, haptoglobin, and transferrin
have a half-life of secretion of 30, 40, and 75 min, respec-
tively (41), and it takes ~30 min for newly synthesized ApoB
100 to be packaged and released into the circulation (42, 43); thus there is a time lag between protein synthesis and appearance in the plasma.

The high sensitivity of this technique allowed us to detect
the effects of fasting on protein synthesis during an 8-h label-
ing experiment and showed that fasting has a differential
effect on protein synthesis in accordance with the biological
function of the protein. Specifically, fasting increased the

Fig. 8. Influence of feeding on synthesis of selected plasma proteins. Rats either had full access to food or fasted overnight before and during 2H2O administration. The red and blue dots represent the observed synthesis rate for fasted and fed rats, respectively. The solid lines are the regression lines, and the dashed lines are the 95% confidence limits for the mean. The slope of M1 labeling of proteolytic peptides, body water labeling, and the asymptotic number of incorporated 2H(N) were used for calculation of the FSR.
synthesis rate of ApoB 100, whereas albumin and fibrinogen synthesis were reduced. The divergent effect of fasting on lipoprotein ApoB 100 and albumin synthesis is in agreement with previous reports (44, 45). Stimulated synthesis of ApoB 100 suggests very low density lipoprotein secretion induced in response to an increased energy requirement of muscle and heart for very low density lipoprotein-bound TG. However, the synthesis of albumin, the most abundant plasma protein, is regulated by amino acid substrate availability in normal conditions (46). Although it is not clear why fasting reduces fibrinogen synthesis, a similar reduction in fibrinogen synthesis was observed in healthy human subjects after a short term increase in plasma free fatty acids by lipid infusion (47). We speculate that the increase in fibrinogen synthesis in fasted rats may be due to stimulated lipolysis and the consequent elevation of plasma free fatty acids.

In conclusion, we demonstrated the development of a $^2$H$_2$O metabolic labeling technique and outline a rationale for its application in broad scale studies of proteome dynamics in vivo. Because of magnification of labeling in proteolytic peptides, this technique allows measurement of protein synthesis in a few hours, which cannot be achieved by previous methods. As a safe nonradioactive tracer, $^2$H$_2$O is routinely used in human studies, and therefore the $^2$H$_2$O metabolic labeling technique presented herein could be applied to study proteome dynamics in humans. In addition to proteins, $^2$H$_2$O metabolic labeling results in $^2$H incorporation into other biological molecules; thus this technique can be expanded to study dynamic genome, proteome, and metabolome interactions.

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The data are expressed as the means ± S.E.

Table VI: FSR in short term experiment and the effect of nutritional status on protein synthesis

| Protein name | Peptide sequence | Observed plateau labeling (%) | Slope of labeling (%/h) | FSR (%/h) |
|--------------|------------------|-------------------------------|-------------------------|-----------|
|              |                  | Fed                           | Fasted                  | Fed       | Fasted   |
| ApoA I       | ADALR            | 29.90 ± 0.39                  | 0.98 ± 0.10             | 1.10 ± 0.08 | 3.36 ± 0.34 | 3.77 ± 0.27 |
|               | AEEFR            | 35.49 ± 0.64                  | 1.78 ± 0.10             | 1.66 ± 0.09 | 4.08 ± 0.26 | 4.47 ± 0.24 |
| ApoA IV      | PLVEDVQSK        | 28.05 ± 0.56                  | 2.99 ± 0.60             | 3.78 ± 0.46 | 11.28 ± 2.26 | 14.26 ± 1.74 |
|               | APLVEDVQSK       | 33.94 ± 1.25                  | 3.94 ± 0.27             | 4.23 ± 0.25 | 10.62 ± 0.73 | 11.40 ± 0.67 |
| ApoB 100     | PAEQTSSWK        | 41.38 ± 2.27                  | 4.62 ± 0.14             | 6.32 ± 0.64 | 11.62 ± 0.35 | 15.90 ± 1.61 |
|               | GFEPTLEALFGK²    | 50.49 ± 2.20                  | 4.93 ± 0.30             | 5.75 ± 1.17 | 9.79 ± 0.60 | 15.03 ± 2.32 |
| ApoE         | GVPAAETR         | 45.82 ± 1.24                  | 4.45 ± 0.26             | 4.20 ± 0.47 | 9.33 ± 0.55 | 8.81 ± 0.99 |
|               | VGAQRQ           | 41.37 ± 1.12                  | 4.05 ± 0.28             | 3.68 ± 0.23 | 10.19 ± 0.70 | 9.26 ± 0.58 |
| ApoH         | PPPPPIP          | 13.39 ± 0.41                  | 0.88 ± 0.03             | 0.88 ± 0.11 | 6.64 ± 0.23 | 6.64 ± 0.83 |
|               | PDELPIAVVPLK     | 38.83 ± 1.13                  | 2.58 ± 0.19             | 2.59 ± 0.14 | 6.49 ± 0.48 | 6.52 ± 0.35 |
| C-reactive protein | SIFSYATK      | 20.50 ± 0.71                  | 0.85 ± 0.02             | 1.07 ± 0.14 | 4.01 ± 0.09 | 5.05 ± 0.66 |
|               | SPNLNWR          | 20.08 ± 0.70                  | 0.55 ± 0.23             | 0.70 ± 0.12 | 2.96 ± 1.13 | 3.77 ± 0.65 |
| Ceruloplasmin | AFFQVR           | 33.02 ± 1.51                  | 1.72 ± 0.09             | 1.94 ± 0.22 | 5.41 ± 0.28 | 6.10 ± 0.69 |
|               | QGER             | 30.66 ± 0.85                  | 1.56 ± 0.09             | 1.50 ± 0.08 | 4.91 ± 0.28 | 4.72 ± 0.25 |
| Haptoglobin  | PTTLLYVGK        | 15.50 ± 0.89                  | 1.48 ± 0.08             | 1.86 ± 0.19 | 9.31 ± 0.50 | 11.70 ± 1.19 |
|               | DWQQTMADK        | 50.06 ± 2.04                  | 4.85 ± 0.46             | 4.20 ± 0.38 | 10.77 ± 1.02 | 9.32 ± 0.84 |
| Transferrin  | SQGCAPGYK        | 44.83 ± 1.34                  | 0.68 ± 0.07             | 0.82 ± 0.04 | 1.43 ± 0.15 | 1.72 ± 0.08 |
|               | TGACQVLKVK       | 46.63 ± 1.88                  | 0.96 ± 0.08             | 1.21 ± 0.25 | 1.91 ± 0.16 | 2.40 ± 0.50 |
| Fibrinogen β polypeptide | GGFTVQTEANK | 70.04 ± 5.01                  | 5.72 ± 0.65             | 1.71 ± 0.52 | 8.30 ± 0.94 | 2.48 ± 0.75 |
|               | ATNEDTkk         | 30.14 ± 3.83                  | 2.41 ± 0.30             | 1.24 ± 0.17 | 8.27 ± 1.03 | 4.25 ± 0.58 |

² The peptide was analyzed by FT-ICR instrument.

⁎ p < 0.05 fed versus fasted. The difference in slopes was assessed by evaluating the interaction term between time and nutritional status.

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