Quantifying apoprotein synthesis in rodents: coupling LC-MS/MS analyses with the administration of labeled water

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Abstract Stable isotope tracer studies of apoprotein flux in rodent models present difficulties as they require working with small volumes of plasma. We demonstrate the ability to measure apoprotein flux by administering either 3H- or 18O-labeled water to mice and then subjecting samples to LC-MS/MS analyses; we were able to simultaneously determine the labeling of several proteolytic peptides representing multiple apoproteins. Consistent with relative differences reported in the literature regarding apoprotein flux in humans, we found that the fractional synthetic rate of apoB is greater than apoA1 in mice. In addition, the method is suitable for quantifying acute changes in protein flux: we observed a stimulation of apoB production in mice following an intravenous injection of Intralipid and a decrease in apoB production in mice treated with an inhibitor of microsomal triglyceride transfer protein. In summary, we demonstrate a high-throughput method for studying apoprotein kinetics in rodent models. Although notable differences exist between lipoprotein profiles that are observed in rodents and humans, we expect that the method reported here has merit in studies of dyslipidemia as i) rodent models can be used to probe target engagement in cases where one aims to modulate apoprotein production and ii) the approach should be adaptable to studies in humans.—Zhou, H., W. Li, S-P. Wang, V Mendoza, R. Rosa, J. Hubert, K. Herath, T. McLaughlin, R. J. Rohm, M. E. Lassman, K. K. Wong, D. G. Johns, S. F. Previs, B. K. Hubbard, and T. P. Roddy. Quantifying apoprotein synthesis in rodents: coupling LC-MS/MS analyses with the administration of labeled water. J. Lipid Res. 2012. 53: 1223–1231.

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In vivo turnover studies have contributed to our understanding of the physiology and pathophysiology of apolipoprotein metabolism. Traditionally, investigators have administered a labeled amino acid (e.g., 13C- or 2H-leucine) and measured its incorporation into various apoproteins, including apoB100 and apoA1 (1, 2). Although useful, the methods are not well suited for application in rodent models. For example, sample preparation methods often require multiple centrifugation steps to separate different lipoprotein fractions, followed by electrophoretic separation of the apoproteins; this typically requires a minimum of several hundred microliters of plasma (3). In addition, investigators determine the flux rates by collecting multiple samples from a given subject and fitting the labeling curves; clearly this is not practical in studies conducted in rodent models in which sample volumes are often a limiting factor (e.g., mice have ~1 ml of blood).

In addition to the concerns noted above regarding the analytical challenges that are associated with studies of lipoprotein kinetics in small animals, one needs to consider the route of administering the tracer. The administration of a labeled amino acid typically requires intravenous access; i.e., tracers are either given as a primed infusion or a bolus injection (1, 2). Although it is feasible to catheterize small animals, the ability to circumvent this step has obvious advantages. Because labeled water (either 2H or 18O) can be given via an intraperitoneal injection, virtually no surgical expertise is needed to administer the tracer (4, 5). In addition, as the t1/2 of water is relatively slow in rodents (e.g., ~2 days for 2H in a mouse), it is possible to maintain a constant labeling of the precursor pool for several hours by administering a single bolus of either water tracer (6), and this can be extended by allowing the animals access to enriched drinking water (4, 7).

Recent studies have demonstrated the ability to quantify protein synthesis using labeled water in rodents and humans (8–13). In this report, we have examined the use of an LC-MS/MS-based method for quantifying the turnover of plasma apolipoproteins in mice given either 2H2O or...
H\textsuperscript{18}O. Attention was focused on quantifying the isotope labeling and measuring the abundance of multiple proteolytic peptides, which, in total, yields an estimate of absolute rates of apoprotein flux in vivo.

**MATERIALS AND METHODS**

Unless noted, chemicals and reagents, including 99.8\% H\textsuperscript{2}O and 97\% H\textsuperscript{18}O, were purchased from Sigma-Aldrich. C57BL/6J mice were purchased from Taconic and fed a standard carbohydrate-based diet. All studies were approved by the Institutional Animal Care and Use Committee of Merck Research Laboratories.

**In vivo labeling studies**

Chronic labeling studies. Male C57BL/6J mice (~12 weeks old) were fed a standard diet ad libitum. Animals were given an intraperitoneal injection of \textsuperscript{2}H\textsubscript{2}O or \textsuperscript{2}H\textsubscript{18}O (20 µl x g\textsuperscript{-1} of body weight), then returned to their cages and maintained on 5\% \textsuperscript{2}H- or \textsuperscript{18}O-labeled drinking water. Mice were euthanized at various points up to 48 h after tracer administration. Plasma samples were frozen until analyses.

Acute labeling studies. Male C57BL/6J mice (~12 weeks old) were fed a standard diet ad libitum. Animals were given an intraperitoneal injection of \textsuperscript{2}H\textsubscript{2}O (20 µl x g\textsuperscript{-1} of body weight) and then euthanized at various points up to 6 h after tracer administration. Plasma samples were frozen until analyses. In a second experiment, mice were studied under conditions that were expected to perturb apoB flux; i.e., control versus Intralipid challenge (50 µl Intralipid 20 per mouse, to stimulate apoB production) versus an inhibitor of microsomal triglyceride transfer protein (MTPi, Pfizer compound CP-346086, at 50 µg per kg, to inhibit apoB production) (14). In those studies, we tested both \textsuperscript{2}H\textsubscript{2}O- and \textsuperscript{3}H\textsubscript{2}O-labeled water in the respective groups (20 µl of either \textsuperscript{2}H\textsubscript{2}O or \textsuperscript{3}H\textsubscript{2}O was administered x g\textsuperscript{-1} of body weight), mice were euthanized 2 h later, and plasma samples were frozen until analyses. The injection of Intralipid was given at the same time as the water tracers (i.e., 2 h before the samples were collected), and the MTPi was given 1 h before the water tracers.

**Analytical studies**

Water labeling. The \textsuperscript{2}H-labeling of plasma water was determined using GC-MS as described by Shah et al. (15). Briefly, \textsuperscript{2}H\textsubscript{2}O was incubated with various proteins and digested with trypsin. The resulting peptides were analyzed using an Agilent DB5-MS column (30 m x 250 µm x 0.15 µm), the oven was initially set at 100°C then programmed to increase at 35°C per min to 250°C, and helium carrier flow was set at 1.0 ml x min\textsuperscript{-1} (2 µl of sample was injected using a 40:1 split). The solution was evaporated under a stream of nitrogen and then dissolved in 150 µl chloroform. Samples were analyzed using a Thermo Orbitrap mass spectrometer coupled with Waters nanoAcquity UPLC using a data-dependent acquisition method. The gradient was 98% A (0.1% formic acid in water) / 2% B (0.1% formic acid in acetonitrile) ramped to 80% A at 16 min, 10% A at 19 min, 98% A at 19.8 min. The column was a Waters Symmetry NanoEase column, 3.5 µm, 300 µm x 50 mm maintained at 50°C, and the flow rate was 10 µl/min.

The acquired LC-MS/MS data files were imported into Eldicator Data Analysis Suite, and the tandem mass spectra were searched against mouse protein database using SEQUEST. Proteins were identified using the following filter criteria: 1+, Xcorr above 3.75; delta Cn above 0.1. Blast searches (NCBI) were performed to ensure that peptide sequences were unique to the target proteins. Isotope labeling was then determined using multiple reaction monitoring of M0, M1, and M2 of the product ions of a given peptide using a Thermo TSQ Quantum triple quadrupole mass spectrometer coupled with Waters nanoAcquity UPLC; the separation condition was identical to that used with the Orbitrap. The mass spectrometric acquisition parameters were as follows: Q1 = 0.7, Q3 = 0.7, scan width = 0.5, scan time = 150 ms, collision energy = 18 V, S-lens = 120.

For protein quantification measurements, 4 µl of plasma was diluted with 136 µl ammonium bicarbonate (pH 8.0), and a known amount of a stable isotope-labeled peptide standard (i.e., 50 µl of 80 nM solution of GFEPT-[\textsuperscript{2}H\textsubscript{10}]LEALFGK; Bachem, Cat.

| Protein | Accession Number | Peptide Sequence Identified | Product Ion | Transitions Monitored |
|---------|-----------------|-----------------------------|-------------|----------------------|
| apoA1   | AAH91745        | VAPLGAELQESAR               | GAEI QESAR  | 670.86–960.47        |
|         |                 |                             | 671.36–961.47 | 761.86–962.47        |
| apoA2   | AAH4358.1       | GFEPTLEALFGK                | PTLLEALFGK  | 654.85–975.55        |
|         |                 |                             | 655.35–976.55 | 655.85–977.55        |
| apoC3   | AAH21776.1      | FTGFDWSPEDQPTPAIES          | PEDQPTPAIES | 1006.49–1183.55      |
|         |                 |                             | 1006.97–1184.55 | 1070.47–1185.55      |
| apoA2   | NP_035092.2     | THEQITPLVR                  | EQITPLVR    | 597.34–965.56        |
|         |                 |                             | 597.84–966.56 | 598.34–967.56        |
| apoC4   | NP_031949.2     | ALVQLEQQR                   | QQLEQQR     | 616.34–948.49        |
|         |                 |                             | 616.84–949.49 | 617.34–950.49        |
| apoE    | AAH28816.1      | TANLGAQAQPLRR               | GAGAAQPLRR  | 620.34–840.47        |
|         |                 |                             | 620.84–841.47 | 621.34–842.47        |

Various protein names and accession numbers are shown, as well as the peptides, product ions, and MS/MS transitions used for estimating the labeling.

The \textsuperscript{15}O-labeling of plasma water was determined using GC-MS as described by Brunengraber et al. (6). Briefly, 5 µl of plasma was reacted with PCL, to generate phosphoric acid, and 150 µl of TMS-diazomethane (Sigma) was then added to generate the trimethylphosphate. The solution was evaporated under a stream of nitrogen and then dissolved in 150 µl chloroform. Samples were analyzed using an Agilent 5973 MS column to a 6890 GC oven fitted with an Agilent DB5-MS column (30 m x 250 µm x 0.15 µm), the oven was initially set at 100°C then programmed to increase at 35°C per min to 250°C, and helium carrier flow was set at 1.0 ml x min\textsuperscript{-1} (2 µl of sample was injected using a 40:1 split), trimethylphosphate was eluted at ~1.9 min, the mass spectrometer was set to perform selected ion monitoring of m/z 140 and 142 (10 ms dwell time per ion) in the electron impact ionization mode.
Calculations

In mice given either $^2$H$_2$O or H$_2^{18}$O, excess labeling is calculated by normalizing the M1 or M2 isotope of a peptide against the M0 isotope [i.e., the isotopically substituted species (singly or doubly, respectively) versus the monoisotopic species] and then subtracting the mean background ratio(s) observed in control mice (i.e., mice that did not receive any isotope) (17). In long-term studies, the fractional synthesis rate (FSR) is calculated from the exponential increase in protein labeling using equation 1:

$$\text{protein labeling}_{t,\text{final}} = \text{final labeling} \times (1 - e^{-\text{FSR} \times t}) \quad (Eq. 1)$$

where $t$ is the time after exposure to $^2$H$_2$O or H$_2^{18}$O and final labeling equals the asymptotic labeling of a proteolytic peptide (which is influenced by the amino acid composition of the peptide and the equilibration of isotope in the respective amino acids) (17). When $^2$H$_2$O is used, we model the change in abundance of M+1-labeled molecules, whereas when H$_2^{18}$O is used, we model the change in the abundance of M+2-labeled molecules.

In short-term studies (e.g., following an acute perturbation with Intralipid or MTPi), the FSR is calculated using equation 2:

$$\text{FSR} = \frac{\text{product labeling}_{t\text{h}}}{\text{precursor labeling}_{t\text{h}}} \times n \quad (Eq. 2)$$

where product labeling$_{t\text{h}}$ represents the total labeling of a proteolytic peptide 2 h postinjection of the respective tracers and $n$ represents the number of copies of the precursor that are incorporated (17–20). For the apoB peptide, we assumed $n = 13$ and $n = 9$ for $^2$H and $^{18}$O, respectively.

As noted, the precursor labeling is influenced by the amino acid composition of the peptide and the equilibration of isotope in the respective amino acids (Fig. 1). Although one can estimate the precursor labeling from the asymptotic (or steady-state) labeling of a protein that is obtained from a long-term study, in cases where $^2$H$_2$O is used acutely, it is difficult to predict the maximum labeling because $^2$H is incorporated into various carbon-bound positions of amino acids (21). Therefore, one requires knowledge regarding the equilibration constants between hydrogen in water and peptide-bound amino acids. We estimated the precursor labeling for $^2$H from previous investigations (21, 22). However, when H$_2^{18}$O is used, we expect that one $^{18}$O atom will be stably bound per peptide bond and additional $^{18}$O atoms for certain amino acid side chains (e.g., glutamine or glutamate) (23–26). We estimated the precursor labeling for $^{18}$O by counting the number of peptide bond oxygens and the side chain oxygens (7). A second approach to estimating $n$ is to run a long-term tracer study and experimentally determine the steady-state labeling. The asymptotic labeling reflects the labeling of the water and $n$, and as we measured the water labeling, we could calculate $n$ (5, 9, 27).

To estimate the absolute flux, one needs to measure the pool size. In studies of apoB metabolism, we estimated its concentration as described above. Therefore, total apoB flux was determined by multiplying equation 2 by the concentration of total apoB.

Statistics

Comparisons were either made using a two-tailed $t$-test (assuming equal variance) or ANOVA with Tukey's posthoc testing.

RESULTS

Because measurements of protein synthesis require reliable quantitation of the isotopic labeling of a given analyte, a critical first step in examining the LC-MS/MS approach centered on determining the precision of the assay(s). For example, Fig. 2 demonstrates the effect of peak intensity on the apparent natural isotopic labeling ratio of the peptide ARPAEDLR (an apoA1-derived tryptic peptide). Although it is clear that signals with lower intensity are generally less reproducible, the coefficient of variation in either the M1/M0 ratio or the M2/M0 ratio can by minimized by increasing the signal intensity. Note that here we have considered a worst-case scenario, as these tests were run by spiking known amounts of ARPAEDLR into plasma digests. Although the precision can be affected by multiple variables, such as coeluting peptides, electronic drifts, etc., similar observations were made when analyzing pure standards (unpublished data), suggesting that the noise in the assay can be minimized by injecting more material.

Figure 3 demonstrates the ability to simultaneously quantify changes in the isotopic labeling of multiple apoproteins following immunodepletion and tryptic digestion of plasma. As is shown, we were able to measure the incorporation of $^2$H and $^{18}$O into peptides derived from apoE, apoB, apoA4, apoC3, apoA2, and apoA1. Regardless of whether mice were given $^2$H$_2$O or H$_2^{18}$O, we

![Fig. 1. Generation of labeled amino acids in the presence of labeled water. In cases where $^2$H$_2$O is administered, the labeling of amino acids largely depends on the equilibration with keto-acids and/or de novo synthesis, which can lead to different levels of incorporation depending on whether the amino acid is essential (e.g., leucine) or nonessential (e.g., alanine). In cases where H$_2^{18}$O is administered, one expects an instantaneous labeling of amino acids during proteolysis; $^{18}$O can be incorporated into some side chain oxygens (e.g., glutamate), which may be back-exchanged during sample preparation. In addition, one expects fairly uniform labeling in cases in which H$_2^{18}$O is administered, as the route of labeling is similar for most amino acids versus amino acid-dependent labeling. In cases where $^2$H$_2$O is administered, the latter occurs because amino acids contain different numbers of carbon-bound hydrogens and each is subject to differential labeling. Note that $^2$H and D are used interchangeably in the figure.](image-url)
observed comparable differences in the relative rates of turnover for the various proteins (i.e., apoE > apoB > apoA1, etc.). Note that the water labeling was maintained at a reasonable steady state in mice given $^{2}$H$_2$O, but there was a slight decrease in the labeling of $^{18}$O in mice given H$_2$18O (inset); this reflects the fact that mice were identically dosed, and as expected, there is a greater turnover of $^{18}$O in body water because oxygen is diluted during the exchange with oxygen in CO$_2$ (7, 28). This lack of steady-state labeling in mice given H$_2$18O does not impact our findings (as noted, the labeling profiles of apoE > apoB > apoA1, etc., in both of the tracer groups); however, this observation implies that studies that require a long-term administration of the tracer(s) should adjust the design(s) to ensure a steady-state water labeling (7) and/or account for changes in the water labeling in the modeling (28). As the labeling of most peptides approached steady-state labeling, it was possible to experimentally estimate the $n$ for the different tracers (Table 2), and those values compare reasonably...

![Fig. 2. Reproducibility of the isotope ratios.](image)

Estimates of protein flux require a measure of the isotope labeling of a given peptide. Various amounts of the mouse apoA1-derived peptide ARPALEDLR were spiked into human plasma digests and M1/M0 (panel A) and M2/M0 (panel B) ratio were measured in five replicates. The insets show the selected ion chromatograms of the peptide at 10 nM. Clearly, there is a strong concentration dependency on the apparent background labeling. Note that reasonable precision was achieved at higher concentrations, i.e., the coefficient of variation < 5% at ≈10$^4$ pM.

![Fig. 3. Labeling of apoproteins during chronic administration of labeled water.](image)

Mice were given a bolus of either $^{2}$H$_2$O or H$_2$18O and then allowed free access to $^{2}$H- or 18O-labeled drinking water, respectively. The isotopic labeling of several apoproteins was determined using samples collected at different time points; the inset demonstrates the labeling of plasma water in the respective groups (data are shown as the average ± SEM, three mice per group per time point, shaded bars represent $^{2}$H$_2$O whereas solid bars represent H$_2$18O). Note that in cases where $^{2}$H$_2$O was administered, the y axis represents the excess labeling in the M1 form of the peptide (open circles) whereas in cases where H$_2$18O water was administered, the y axis represents the excess labeling in the M2 form of the peptide (solid circles). The legend contains the tryptic peptide used in the analyses and identifies the fragment ion that is used to determine the labeling; note that the labeling of the first several amino acids in each sequence (shown in parenthesis) was not determined via the transitions that were monitored (see Table 1).
mating the kinetic constants (it simply requires that one have knowledge of the expected labeling), studies that use \(^2\text{H}_2\text{O}\) should consider peptides that predominantly contain nonessential amino acids to maximize the shift in the labeling.

On the basis of the data shown in Fig. 3, we next designed a pilot experiment to determine the initial labeling profiles of the various apoproteins. For example, Foster et al. (29) have elegantly described the effects of different assumptions when modeling kinetic data. Although the data shown in Fig. 3 are less than ideal for determining the early changes in labeling, we found that the incorporation of \(^2\text{H}\) in some apoproteins approaches a steady state within a few hours postinjection of the tracer, whereas the labeling of others remains in a pseudo steady state (Fig. 4). Note that the time-dependent changes for most proteins are reasonably stable; however, in a few instances, there appears to be an outlying data point (e.g., the 1.5 h sample for apoA1 and the 2 h sample for apoA2). This is not unexpected as the aim of this pilot was to establish the general temporal response; therefore, we euthanized only one animal per time point. On the basis of these observations, we determined that studies of apoB flux, for example, should be run for \(\sim 2\) h. This would ensure that measurements are made during a period in which the change in protein labeling is pseudo-linear and minimizes the error when interpreting data using samples collected at a single time point (see equation 2) (29). Using the data contained in Fig. 4, it was possible to estimate the \(t_{1/2}\) for the various proteins; these values are \(\sim 1.1\) h for apoE, \(\sim 1.4\) h for apoB, \(\sim 3.8\) h for apoA2, \(\sim 10.1\) h for apoA4, \(\sim 16.2\) h for apoC3, and \(\sim 27.7\) h for apoA1. Note that the values for apoE, apoB, and apoA2 were determined by fitting the labeling curves using equation 1, whereas the values reported in Table 2. Number of labeling sites in a given peptide (\(n\)) can be inferred on consideration of a peptide’s amino acid composition and/or via direct experimentation.

| Protein | Theoretical n \(^2\text{H}_2\text{O}\) | Experimentally Determined n \(^2\text{H}_2\text{O}\) | Theoretical n \(^2\text{H}_2\text{O}\) | Experimentally Determined n \(^2\text{H}_2\text{O}\) |
|---------|---------------------------------|-----------------------------------|---------------------------------|-----------------------------------|
| apoA1   | 29                              | 11                                | 34                              | 11                                |
| apoB    | 12                              | 9                                 | 13                              | 9                                 |
| apoC3   | 26                              | 12                                | 29                              | 12                                |
| apoA2   | 16                              | 8                                 | 18                              | 7                                 |
| apoA4   | 19                              | 9                                 | 16                              | 7                                 |
| apoE    | 27                              | 9                                 | 26                              | 8                                 |

Theoretical values for the number of \(^2\text{H}\) or \(^{18}\text{O}\) were estimated using published data (see Refs. 7, 21, and 22), whereas the experimentally determined values were derived by fitting the data obtained from the chronic labeling study (i.e., those contained in Fig. 3), the asymptotic labeling of a given peptide, and the water labeling used for that purpose. In most cases, there is reasonable agreement between the different values.

well with previously published values for the labeling of individual amino acids (7, 21, 22).

The data shown in Fig. 3 emphasize another important concept regarding the incorporation of labeled water into a protein. For example, the labeling of apoE is rather striking in that there is a major difference between the steady-state enrichment in mice given \(^2\text{H}_2\text{O}\) versus those given \(^2\text{H}_2\text{O}\). This is expected given the amino acid sequence and the nature of how the different isotopes are incorporated. In cases where \(^2\text{H}_2\text{O}\) is administered, one expects a more uniform incorporation of the label (e.g., primarily in the peptide bonds), whereas when \(^2\text{H}_2\text{O}\) is given, one expects the asymptotic labeling to be strongly affected by the presence of several glycine and alanine residues (each containing multiple \(^2\text{H}\) from body water). Although the variable labeling does not constitute a problem when estimating the kinetic constants (it simply requires that one have knowledge of the expected labeling), studies that use \(^2\text{H}_2\text{O}\) should consider peptides that predominantly contain nonessential amino acids to maximize the shift in the labeling.

On the basis of the data shown in Fig. 3, we next designed a pilot experiment to determine the initial labeling profiles of the various apoproteins. For example, Foster et al. (29) have elegantly described the effects of different assumptions when modeling kinetic data. Although the data shown in Fig. 3 are less than ideal for determining the early changes in labeling, we found that the incorporation of \(^2\text{H}\) in some apoproteins approaches a steady state within a few hours postinjection of the tracer, whereas the labeling of others remains in a pseudo steady state (Fig. 4). Note that the time-dependent changes for most proteins are reasonably stable; however, in a few instances, there appears to be an outlying data point (e.g., the 1.5 h sample for apoA1 and the 2 h sample for apoA2). This is not unexpected as the aim of this pilot was to establish the general temporal response; therefore, we euthanized only one animal per time point. On the basis of these observations, we determined that studies of apoB flux, for example, should be run for \(\sim 2\) h. This would ensure that measurements are made during a period in which the change in protein labeling is pseudo-linear and minimizes the error when interpreting data using samples collected at a single time point (see equation 2) (29). Using the data contained in Fig. 4, it was possible to estimate the \(t_{1/2}\) for the various proteins; these values are \(\sim 1.1\) h for apoE, \(\sim 1.4\) h for apoB, \(\sim 3.8\) h for apoA2, \(\sim 10.1\) h for apoA4, \(\sim 16.2\) h for apoC3, and \(\sim 27.7\) h for apoA1. Note that the values for apoE, apoB, and apoA2 were determined by fitting the labeling curves using equation 1, whereas the values reported

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![Fig. 4. Acute labeling of apoproteins.](image-url)

To estimate the early time course for protein labeling, mice were given a single bolus of \(^2\text{H}_2\text{O}\), and the incorporation of \(^2\text{H}\) into various apoproteins was measured (\(n = 1\) animal per time point). It is clear that some proteins (e.g., apoB and E) reach a steady-state labeling within the experimental window, whereas most others demonstrate a pseudo-linear change in the labeling. Note that each data point represents a single animal. The \(^2\text{H}\)-labeling of water was stable (\(\sim 2.5\%\) excess) during the time course of this study (not shown).
TABLE 3. Parameters used to estimate apoB flux

| Parameter                  | Control | Intralipid | MTPi | Control | Intralipid | MTPi |
|----------------------------|---------|------------|------|---------|------------|------|
| Water labeling (% excess)  | 2.5 ± 0.1| 2.7 ± 0.1  | 2.7 ± 0.1| 2.7 ± 0.1| 2.8 ± 0.2  | 2.9 ± 0.4|
| apoB labeling (% excess)   | 5.1 ± 0.4| 5.9 ± 0.6  | 3.3 ± 0.5  | 3.3 ± 0.5  | 4.7 ± 0.2  | 2.9 ± 0.2*  |
| Fractional synthesis rate (per 2 h) | 0.182 ± 0.012 | 0.212 ± 0.012* | 0.102 ± 0.016* | 0.208 ± 0.018 | 0.248 ± 0.013* | 0.122 ± 0.014*  |
| apoB concentration (nM)    | 323 ± 13 | 459 ± 41*  | 155 ± 12*  | 340 ± 15  | 500 ± 44*  | 187 ± 33*  |
| apoB production (nM per 2 h)| 54 ± 5  | 82 ± 7*    | 16 ± 3*    | 57 ± 6    | 84 ± 8*    | 18 ± 2*   |

All measurements were made 2 h postinjection of the tracers (data shown as average ± SEM, five mice per group). Rates of apoB production were determined using Equation 2 (see Materials and Methods); n = 13 and n = 9 in cases where ²H₂O and H₂¹⁸O were administered, respectively. A t-test was used to compare a given treatment with the different tracers, e.g., ²H₂O-control versus H₂¹⁸O-control (no differences were observed). ANOVA (Tukey’s posthoc testing) was used to determine differences within a given tracer group.

αP < 0.05 versus controls.

βP < 0.05 Intralipid versus MTPi.

for apoA4, apoC3, and apoA1 were calculated using equation 2 and using the experimentally determined n (Table 2) in the denominator.

Because the control of apoB production is affected by various factors, we designed an experiment to determine whether our method could detect increases and/or decreases in apoB production. Table 3 contains the requisite parameters that should enable routine investigations in such models. An additional parameter that is required is n, which we derived using previously published data (7, 21). The data contained in Table 3 and Fig. 5 demonstrate that our approach has the ability to detect acute changes in protein flux. For example, the administration of an intravenous bolus of Intralipid led to a stimulation of apoB production, whereas treatment with MTPi led to a decrease in apoB production. These observations were consistently observed using ²H₂O and H₂¹⁸O.

DISCUSSION

Historically, most studies of lipoprotein kinetics were performed in humans and/or large animals. Although the use of stable isotope tracers has been limited in small animals (e.g., mice) (30), we have demonstrated an approach that should enable routine investigations in such models. The ability to translate knowledge regarding physiology across models may not be obvious in all cases. For example, although there are well-described differences in enzyme activities and lipoprotein profiles between rodents and humans, rodents can be used to address certain questions, especially those regarding the ability to modulate a target. Two central challenges that required attention included i) how to implement a simple tracer protocol and ii) how to measure changes in isotopic labeling of a protein. We consider the novelty of our approach in the context of those challenges.

First, considerable progress has been made regarding working with small animals. In addition to the fact that one can purchase catheterized animals for studies, many academic centers support mouse metabolic phenotyping activities, including several National Institutes of Health (NIH)-funded sites that are expected to serve as core facilities for the general scientific community (see www.mmpc.org). Although it is now somewhat easy to execute studies that require several hours of a tracer infusion, the ability to circumvent this step would allow for higher throughput studies by minimizing the cost/time associated with implanting catheters and limiting the need to ship mice to specialized labs.

We and others have demonstrated the ability to study protein synthesis by administering labeled water (7–9, 12, 31, 32). For example, because subjects will generate ²H- or ¹⁸O-labeled amino acids in the presence of either ²H- or ¹⁸O-labeled water, rates of protein synthesis can be determined by measuring the incorporation of the respective ²H- or ¹⁸O-labeled amino acids into a protein of interest (Fig. 1). In cases where ²H₂O is administered, one expects that the amino acid composition of the protein of interest...
(or proteolytic peptide) will affect the degree of labeling as individual amino acids reach different steady-state labeling. However, as we recently demonstrated (21), it appears that one can reliably interpret the data because in the presence of $^2\text{H}_2\text{O}$, the rate of $^2\text{H}$-labeling of amino acids is relatively fast. Although one expects that exposure to $^2\text{H}_2\text{O}$ will also rapidly label amino acids, it is presumed that $^2\text{H}^{18}\text{O}$ will lead to more uniform labeling of proteolytic peptides, especially when considering the mechanism(s) by which $^{18}\text{O}$ labels amino acids (Fig. 1) (23–26). $^{18}\text{O}$ is incorporated into amino acids during proteolytic cleavage, amino acid activation (not shown in the scheme), and de novo synthesis. In total, this should result in a rapid and stable $^{18}\text{O}$-labeling of free amino acids.

As we previously discussed, the degree of labeling that is observed in a given protein depends on (i) the amount of labeled water that is administered, (ii) the amino acid composition of a given peptide (or fragment ion), and (iii) the synthetic rate (21). In cases where $^2\text{H}_2\text{O}$ or $^2\text{H}^{18}\text{O}$ are administered, one typically expects to observe the incorporation of multiple copies of the precursor in a product; i.e., because the protein acts as a biopolymer of the precursor, the labeling of the protein can exceed that of the precursor (18). This is most obvious in Figs. 3 and 4, in which the labeling of apoE reaches $\approx20\%$ excess $^2\text{H}$ and the body water labeling is $\approx2.5\%$ excess $^2\text{H}$. The apoE peptide that we have monitored contains several amino acids that carry more than one $^2\text{H}$ from body water (e.g., glycine $\sim2$, alanine $\sim4$, etc.) (Table 2). As apoE is known to display a reasonably short $t_{1/2}$, one expects that shifts in its labeling will be the most apparent under these conditions, in contrast to apoA1, which typically has a slower $t_{1/2}$ (1, 33). In cases where one obtains a time course of labeling, it is possible to use equation 1 and neglect $n$ (i.e., the number of labeled sites); however, in short-term studies and/or cases in which a single sample is obtained, it is necessary to account for $n$, so equation 2 is required (18–20).

Note that there is a discrepancy in the literature regarding apoB kinetics: it is not immediately clear whether the liver directly makes VLDL-apoB and LDL-apoB or whether LDL-apoB is made via delipidation (34). Our analysis is rather simplistic and circumvents these concerns; as noted, we are quantifying total apoB flux. In cases where Intralipid is acutely administered via intravenous injection, one might expect a sizeable increase in the concentration of circulating lipids. The dose of Intralipid administered in this study delivered $\sim10\,\text{mg}$ of lipid, yet the endogenous triglyceride pool was on the order of $\sim1.5\,\text{mg}$. Presumably, a fraction of the Intralipid bolus was cleared by the liver, re-packaged into VLDL particles, and then exported; therefore, the stimulation of apoB production that we observed in mice treated with Intralipid is consistent with what one might expect. Likewise, the administration of a known inhibitor of triglyceride secretion (Pfizer compound CP-346086) led to a marked decrease in the production of apoB, again consistent with what one might expect (Table 3 and Fig. 5).

Although we were intrigued to find significant changes in the FSRs of apoB following the administration of Intralipid or MTPi (Table 3 and Fig. 5), it is necessary to consider how the absolute rate of protein synthesis depends on knowledge of the time-course of change in protein concentration. To this point, our interpretation has neglected any effect on protein clearance (or degradation), and this is especially important in mice given a bolus of Intralipid (35, 36). For example, based on the increase in the concentration of apoB (Table 3), it is clear that the Intralipid challenge led to an imbalance between production and removal; the concentration increased by $\sim145\,\text{nM}$ in 2 h (i.e., $\sim330\,\text{nM}$ in control versus $\sim475\,\text{nM}$ in Intralipid, regardless of whether animals were given $^2\text{H}_2\text{O}$ or $^2\text{H}^{18}\text{O}$). Assuming, as we have done, that the concentration rapidly changed to a new steady state suggests that $\sim30\,\text{nM}$ of protein was newly made (i.e., $\sim50\,\text{nM}$ in controls versus $\sim80\,\text{nM}$ in Intralipid, regardless of the tracer). From this simple calculation, it is obvious that there is a substantial gap between the change in concentration and the apparent change in the absolute rate of production, $\sim145\,\text{nM}$ and $\sim30\,\text{nM}$, respectively. As we did not collect samples to describe the exact shape of the curve, it is not possible to make firm statements regarding explicit changes to the absolute rate of protein synthesis and/or degradation (e.g., did the concentration reach a new steady state within the first 15 min of the Intralipid bolus, or did the concentration rise steadily over the 2 h?) We believe that in the absence of data which demonstrate the time-dependent change in apoB concentration, it is better to interpret the absolute protein flux results with caution. It is clear that Intralipid stimulated the fractional rate of synthesis, which presumably translated into a stimulation of the absolute rate of protein synthesis; however, the exact magnitude of the change in the absolute rate is not clear. In total, our observations suggest that it is possible to determine the acute impact of a perturbation on protein synthesis and protein breakdown provided that one collect the appropriate samples to definitively interpret the data, and we expect that the methods reported here can be used for such purposes.

Finally, it is of interest to consider the effect of timing on the studies reported here and differences between our design and many articles in the literature. Investigators often collect numerous samples from a given subject to determine protein flux. For example, during a typical human study, one may obtain more than 10 samples per subject (1, 2). Clearly, this is not practical in small animals and may not be necessary in humans either (29). As we have demonstrated in rodent models, it is possible to use a single sample to estimate protein flux. Although this is appealing, especially as it minimizes the number of samples that are required for analyses, one must carefully choose when to collect a sample. As outlined by Foster et al. (29), the sampling interval can have substantial consequences on the apparent turnover rate of a given protein. Although we have demonstrated the ability to study apoB flux by sampling $\sim2\,\text{h}$ postracer injection, studies focused on proteins with faster or slower half-lives would likely require additional consideration. The consequences are that one may underestimate the $t_{1/2}$ if a protein turns over rapidly.
and samples are collected later (29) and that the analytical window is compromised when measuring the labeling of a protein with a long t_{1/2} if it is sampled too early. In addition, it is often necessary to add time delays in the modeling to account for packaging of newly made proteins before they are secreted (29). Although we recognize that we neglected to include such a delay, we do not believe that it has a serious impact on our data. For example, although the rates of apoB production may not reflect the true rates of apoB production, we were able to detect the expected directional changes in apoB production (Fig. 5).

In summary, we have demonstrated a simple and robust method for quantifying apoprotein flux in small animals. This method is well suited for high-throughput studies in model systems and can be used to evaluate the efficacy of novel compounds capable of modulating specific targets (e.g., apoB production in mice). The fact that virtually data that we recently obtained regarding the equilibration of 2H-labeling in mice following the administration of 3H_{2}O (21). Although both 2H_{2}O and H_{2}O appear to yield comparable results regarding protein synthesis, 2H_{2}O offers advantages in cases where one also aims to examine lipid flux (37, 38). As both types of labeled water can be given to humans (10, 39), we suspect that it is possible to translate aspects of this work to clinical investigations.

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