**2H₂O-Based High-Density Lipoprotein Turnover Method for the Assessment of Dynamic High-Density Lipoprotein Function in Mice**

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**Objective**—High-density lipoprotein (HDL) promotes reverse cholesterol transport from peripheral tissues to the liver for clearance. Reduced HDL-cholesterol (HDLc) is associated with atherosclerosis; however, as a predictor of cardiovascular disease, HDLc has limitations because it is not a direct marker of HDL functionality. Our objective was to develop a mass spectrometry–based method for the simultaneous measurement of HDLc and ApoAI kinetics in mice, using a single 2H₂O tracer, and use it to examine genetic and drug perturbations on HDL turnover in vivo.

**Approach and Results**—Mice were given 2H₂O in the drinking water, and serial blood samples were collected at different time points. HDLc and ApoAI gradually incorporated 2H, allowing experimental measurement of fractional catabolic rates and production rates for HDLc and ApoAI. ApoE−/− mice displayed increased fractional catabolic rates (P<0.01) and reduced production rates of both HDLc and ApoAI (P<0.05) compared with controls. In human ApoAI transgenic mice, levels and production rates of HDLc and human ApoAI were strikingly higher than in wild-type mice. Myriocin, an inhibitor of sphingolipid synthesis, significantly increased both HDLc flux and macrophage-to-feces reverse cholesterol transport, indicating compatibility of this HDL turnover method with the macrophage-specific reverse cholesterol transport assay.

**Conclusions**—2H₂O-labeling can be used to measure HDLc and ApoAI flux in vivo, and to assess the role of genetic and pharmacological interventions on HDL turnover in mice. Safety, simplicity, and low cost of the 2H₂O-based HDL turnover approach suggest that this assay can be scaled for human use to study effects of HDL targeted therapies on dynamic HDL function. (Arterioscler Thromb Vasc Biol. 2013;33:1994-2003.)

**Key Words:** apolipoprotein A-I ■ cholesterol ■ deuterium oxide ■ lipoproteins, HDL ■ mass spectrometry ■ protein biosynthesis
uptake and turnover.\textsuperscript{15,16} This is a cumbersome method involving isotope labeling of HDL from the donor and injection to the recipient animal after dual radioactive labeling.\textsuperscript{17} Either bolus\textsuperscript{18} or primed/constant infusion of proteogenic (amino acids) and lipogenic (acetate) or cholesterol tracers were used to estimate the synthetic rate of ApoAI (and other apolipoproteins) and lipids in the lipoproteins of interest.\textsuperscript{19} Combinations of stable isotopes, [\textsuperscript{13}C]-acetate and [\textsuperscript{2}H\textsubscript{1}],-[\textsuperscript{2}H\textsubscript{2}]-leucine as labeled precursors of cholesterol/cholesterol ester and ApoAI, respectively, have been used to study HDL turnover in humans.\textsuperscript{12} Aside from technical intricacies, these methods require large amounts of expensive tracers.

In this study, we used an alternative heavy water ([\textsuperscript{2}H\textsubscript{2}O]-based metabolic labeling approach to measure HDL turnover. As a nonradioactive, safe, and low-cost tracer, [\textsuperscript{2}H\textsubscript{2}O] has been widely used to study lipid, protein, and DNA turnover in free living organisms, including humans.\textsuperscript{20–22} [\textsuperscript{2}H\textsubscript{2}O] rapidly equilibrates with total body water (including intracellular fluids), and it readily labels lipogenic precursors, for example, acetyl-CoA/NADPH for lipid synthesis. Application of [\textsuperscript{2}H\textsubscript{2}O] as a tracer for cholesterol synthesis is well established.\textsuperscript{23} Recently, we demonstrated that the steady-state [\textsuperscript{2}H\textsubscript{2}O]-labeling of most intracellular amino acids was achieved in rats within 30 minutes of an intraperitoneal bolus of [\textsuperscript{2}H\textsubscript{2}O], suggesting that the transfer of amino acids to the polypeptide chain is the rate limiting step in protein biosynthesis.\textsuperscript{24} In addition, we have shown that amino acids labeling remained at steady state throughout 60-day labeling experiment in rodents.\textsuperscript{25} These studies validated the assumption that [\textsuperscript{2}H\textsubscript{2}O] is a true tracer precursor for protein synthesis. We have used this approach to quantify the rate of synthesis of plasma lipids, lipoproteins, and acute response proteins under normal and interfibrillar mitochondria.\textsuperscript{29} Because [\textsuperscript{2}H\textsubscript{2}O] incorporates into both cholesterol\textsuperscript{23} and ApoAI,\textsuperscript{24} here, we used [\textsuperscript{2}H\textsubscript{2}O] as a single tracer to study HDL kinetics. In contrast to existing methods, [\textsuperscript{2}H\textsubscript{2}O]-based HDL turnover approach relies on proteomic analyses and allows discrimination and quantification of both human and mouse ApoAI kinetics individually. We applied this method to assess HDL dynamics in ApoE\textsuperscript{−/−} and ApoAI transgenic mice. Furthermore, we applied the HDL turnover method for the evaluation of HDL function simultaneously with an assay for macrophage-specific RCT method, allowing us to compare the results from these 2 assays that track different pools of cholesterol and different aspects of HDL metabolism. Finally, we assessed the effect of myriocin, an inhibitor of sphinogolipid synthesis and modifer of HDL metabolism,\textsuperscript{30} on HDL turnover and RCT. Myriocin treatment increased both HDL flux and macrophage-RCT, suggesting that [\textsuperscript{2}H\textsubscript{2}O]-based HDL turnover analysis may be used as a safe approach to measure HDL flux in humans and gain insight into factors relevant to RCT.

**Materials and Methods**

Materials and Methods are available in the online-only Supplement.

**Results**

**Validation Study**

The key assumption in [\textsuperscript{2}H\textsubscript{2}O]-based metabolic experiment is that [\textsuperscript{2}H\textsubscript{2}O] rapid labels body water and transfers [\textsuperscript{2}H] from [\textsuperscript{2}H\textsubscript{2}O] to [\textsuperscript{2}H]-labeled amino acids, which incorporate into proteins, including ApoAI. This approach also excludes any possibility of ApoAI labeling through hydrogen/deuterium exchange in plasma with [\textsuperscript{2}H\textsubscript{2}O] after being secreted. In our previous studies, we validated the assumption that tissue amino acids are rapidly labeled and attain a steady state.\textsuperscript{24,25,27} The possibility of postsynthetic hydrogen/deuterium exchange was tested through the following experiment. Two ApoB-depleted HDL samples were isolated from untreated wild-type mice plasma. One of the samples (control) was saved until the analyses. The other sample was mixed with 10% [\textsuperscript{2}H\textsubscript{2}O] enriched water and incubated in a slow shaker for 7 days at room temperature. HDL, including ApoAI, were precipitated with 1 mL of cold acetone (−20ºC) from control and [\textsuperscript{2}H\textsubscript{2}O] treated, and proteins were analyzed as described below. The isotopic distribution of tryptic ApoAI peptide VAPLGAEQLQESAR (analyzed as M+2 ion with the m/z of 670.87) was analyzed to assess hydrogen/deuterium exchange with [\textsuperscript{2}H\textsubscript{2}O]. No measurable [\textsuperscript{2}H]-labeling was detected in tryptic ApoAI peptides, suggesting that the [\textsuperscript{2}H]-enrichment of HDL is exclusively related to metabolic labeling but not to hydrogen/deuterium exchange in plasma (Figure I in the online-only Data Supplement).

**HDL Turnover Studies in ApoE\textsuperscript{−/−} and ApoAI\textsubscript{tg/tg} Mice**

We previously demonstrated that bolus of [\textsuperscript{2}H\textsubscript{2}O] rapidly equilibrates with the total body water and intracellular amino acids within 30 minutes.\textsuperscript{24} In this study, we found that intraperitoneal bolus loading of [\textsuperscript{2}H\textsubscript{2}O] (22 [\textmu]L/g body weight) followed with free access to drinking water enriched with [\textsuperscript{2}H\textsubscript{2}O] (6%) for up to 7 days maintains the body water at a steady-state labeling of ≈3.4%. No adverse effects on growth or food consumption were observed attributable to [\textsuperscript{2}H\textsubscript{2}O] administration.

We used [\textsuperscript{2}H\textsubscript{2}O]-metabolic labeling coupled with a mass spectrometry approach to assess HDLc and ApoAI turnover in mice. This approach is based on the rationale that after equilibration with total body water and all cell compartments, [\textsuperscript{2}H\textsubscript{2}O] readily labels acetyl-CoA and NADPH for cholesterol synthesis and amino acids for protein synthesis, respectively. After isolation of ApoB-depleted plasma, lipids were extracted, and total HDLc and ApoAI were analyzed by gas chromatography mass spectrometry and high resolution liquid chromatography tandem mass spectrometry, respectively. The integrated peak ratios of HDLc/[\textsuperscript{2}H\textsubscript{1}],-cholesterol internal standard and an endogenous ApoAI peptide/heavy isotope-labeled internal standard were used for quantification of the absolute amounts of HDLc and ApoAI. The changes in isotopomer distribution of HDLc and proteolytic ApoAI peptides at different time points were used to estimate HDLc and ApoAI fractional catabolic rates (FCR), production rate (PR), and half life (t\textsubscript{1/2}; Figure II in the online-only Data Supplement). We performed a pilot experiment with 1 wild-type mouse using minimal volume blood sampling (25 [\mu]L plasma) at selected time points and preparation of ApoB-depleted plasma followed by mass...
spectrometry analyses to measure HDLc (Figure IIIA in the online-only Data Supplement) and ApoAI turnover (Figure IIB and IIC in the online-only Data Supplement) as described above. ApoAI was analyzed using 3 different peptides. As expected each peptide reaches different asymptotic labeling because sequences are different (Figure IIB in the online-only Data Supplement). The maximal (plateau) labeling of a peptide depends on total body water labeling and the asymptotic number of exchangeable C-bound H atoms. Previously, we have determined the asymptotic number of exchanged H atoms for each proteogenic amino acid and demonstrated that nonessential amino acids, alanine, glutamate, glutamine, glycine, and serine are extensively labeled, meaning that they incorporate a higher number of $^3$H. Small, in some cases negligible, amounts of $^2$H label were found in most essential amino acids through transamination reaction. Thus, peptides TQVQSVIHKASLTQ and VAPLGAELQESAR with several alanine (A), glutamine (Q), and glutamate (E) have higher labeling than that of peptide DFANVYVDAVKV that has only 2 alanine and multiple essential amino acids (Figure IIB in the online-only Data Supplement). However, normalization of labeling at all time points to the maximum labeling for each peptide illustrates that they overlay and yield similar rate constants. Thus, we determined that we could accurately measure the kinetics of ApoAI and HDLc in a single animal, and that the turnover kinetics of the 3 independent ApoAI peptides were similar (coefficient variation < 5%), with $t_{1/2}$ values of ≈19 hours (Figure II in the online-only Data Supplement), similar to previous reports based on radioactive techniques. The $t_{1/2}$ of HDLc was ≈30 hours. Based on this pilot, we chose to use the most abundant endogenous VAPLGAELQESAR peptide and its stable isotope-labeled synthetic analog VAPLV($^{13}$C$_6$)GAEL($^{13}$C$_6$)QESAR (as the internal standard) for our subsequent mouse ApoAI turnover studies.

Because ApoE-deficient mice are known to have lower HDLc levels compared with wild-type mice, we applied this technique for a systematic comparison of HDL turnover in 12-week-old female mice (n=6 of each strain). Consistent with previous studies, the total plasma cholesterol levels were in 12-week-old female mice (n=6 of each strain). Consistent with previous studies, the total plasma cholesterol levels were significantly lower in ApoE−/− mice (P<0.05); however, HDLc and ApoAI levels were significantly lower in ApoE−/− mice compared with wild-type controls (P<0.05; Table 1). HDL turnover analysis revealed that this was accompanied with by an almost 2-fold increase in HDLc and ApoAI FCR (P<0.05; Table 1; Figure 1A and 1B), indicating shorter half lives. In addition, HDLc and ApoAI PR in ApoE−/− mice were significantly lower than that in wild-type mice (2-fold and 1.4-fold, respectively; P<0.05; Table 1). Reverse transcriptase polymerase chain reaction analysis revealed that despite lower plasma ApoAI levels and ApoAI PR in ApoE−/− mice, mRNA of hepatic ApoAI in these animals were similar to wild-type controls (Figure IVA in the online-only Data Supplement). Combined together, these findings suggest that ApoAI levels might be regulated by the post-transcriptional control of translation or post-translational modification, including by the degradation as it was demonstrated in this study. In contrast, hepatic SR-B1 mRNA and protein levels ascertained by reverse transcriptase polymerase chain reaction and Western blot revealed that higher turnover of HDL in ApoE−/− mice was associated with increased expression of SR-B1, the major receptor for hepatic uptake of HDL (Figure 2A and 2B). The increased HDL turnover and SR-B1 expression in ApoE−/− mice suggest that they may have higher hepatic HDLc uptake. Thus, our $^2$H$_2$O-based turnover study demonstrated that ApoE deletion in mice significantly affected HDL metabolism.

To assess the effect of human ApoAI overexpression on HDL turnover, we also performed a separate $^2$H$_2$O-based HDL turnover study on 6 adult male ApoAI transgenic mice. Body water labeling was relatively stable during the 7 days of labeling experiment (Figure V in the online-only Data Supplement). HDLc levels in these mice were 159±45 mg/dL, and as expected much higher than that observed in wild-type mice (53±17 mg/dL, wild-type mouse data reported in Table 1 but no statistics are presented because these were separate experiments performed in different sexes). Proteomic analyses identified both murine and human ApoAI in the HDL of ApoAI transgenic mice. Consistent with previous reports, the plasma level of murine ApoAI (0.44±0.06 mg/dL) was markedly decreased in ApoAI transgensics compared with wild-type mice (95.9±12.3 mg/dL). As expected, because of the sequence differences of murine and human ApoAI peptides, these peptides reach different steady-state $^2$H-enrichment. The $^2$H$_2$O-based kinetic analysis revealed that the FCR of HDLc in human ApoAI transgenic mice (Figure 3A) versus wild-type mice (Table 1) was not altered (2.5±0.4%/h versus 2.7±0.7%/h, respectively). Our method allowed the simultaneous measurement of the turnover of both human and murine ApoAI. The low levels of murine ApoAI in the transgenic mice were associated with both a 10-fold increase in FCR compared with wild-type mice, and a 10-fold decrease in PR versus wild-type mice (compare Figure 3B with Table 1). These results are consistent with the results from previous HDL turnover studies using dual radioisotope approach, suggesting that displacement of mouse ApoAI by human protein in ApoAI transgenic mice leads to increased renal clearance of mouse ApoAI. Reduced PR of murine ApoAI was also paralleled with 4-fold reduction of hepatic ApoAI mRNA determined by reverse transcriptase

### Table 1. Plasma Levels and Kinetics of HDL-Cholesterol and ApoAI in Mice HDL (n=6)

<table>
<thead>
<tr>
<th>Mice</th>
<th>Total Cholesterol</th>
<th>HDL Cholesterol</th>
<th>ApoAI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Levels, mg/dL</td>
<td>Levels, mg/dL</td>
<td>Levels, mg/dL</td>
</tr>
<tr>
<td>Wild type</td>
<td>111.6±22.7</td>
<td>52.8±17.2</td>
<td>95.9±12.3</td>
</tr>
<tr>
<td>ApoE−/−</td>
<td>493.0±97.5</td>
<td>17.5±4.6</td>
<td>32.7±8.5</td>
</tr>
<tr>
<td><strong>P value</strong></td>
<td>&lt;0.0001</td>
<td>0.001</td>
<td>0.0001</td>
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</table>

FCR indicates fractional catabolic rates; HDL, high-density lipoprotein; and PR, production rates.
polymerase chain reaction in these animals (Figure IVB in the online-only Data Supplement), suggesting feedback inhibition of mouse ApoAI production attributable to increased expression of human protein in these animals. This finding contradicts a previous report, which based on Northern blot analyses concluded that mouse ApoAI expression is identical in transgenic and wild-type animals. The FCR of human ApoAI in the transgenic mice was 11.1±0.6%/h (Figure 3B). Normalization of the data at all time points for the maximum plateau labeling of mouse and human ApoAI peptides illustrates clear differences in the turnover rates of mouse and human protein (Figure VI in the online-only Data Supplement). The PR of human ApoAI in the transgenic mice was very high (46.9.0±5.8 mg/kg per hour; Figure 3B), indicating that the elevated plasma HDLc in these mice is a direct consequence of the production of more HDL particles.

**HDL Turnover and Macrophage-Specific RCT Studies in Wild-Type Mice: Effect of Myriocin**

To investigate the relationship between HDL turnover and RCT, we used myriocin as a modifier of sphingolipid and HDL metabolism. We used the 2H2O-metabolic labeling approach and the macrophage-specific RCT method in the same animals during a 3-day time course. This study, in contrast to the previous studies, had 2 minor differences: (1) a lower level of 2H2O enrichment of body water was used; and (2) a shorter 3-day time course was used to be compatible with the RCT study. Myriocin consumption in diet (≈0.3 mg/kg body weight per day) for 2 weeks did not affect food intake or body weight. Body water enrichment was stable and equivalent in the control and myriocin-treated groups at ≈1.6% (Figure VII in the online-only Data Supplement). Although myriocin led to ≈15% increases in total plasma cholesterol and HDLc, these changes were not statistically significant (Table 2). However, the HDLc

Figure 1. High-density lipoprotein (HDL) turnover in ApoE-deficient (triangular symbols) and wild-type (square symbols) mice assessed with 2H2O-metabolic labeling technique. Intraportal bolus loading followed by free access to drinking water enriched with 2H2O (8%) led to a steady-state body water labeling of ≈3.4%. Time course enrichment of 2H incorporation into HDL cholesterol (HDLc; A) and ApoA1 (B). Data show mean±SD, n=6 per group.

Figure 2. Effect of ApoE deletion on hepatic scavenger receptor type B1 (SR-B1) expression. A, Reverse transcriptase polymerase chain reaction of hepatic SR-B1 mRNA (mean±SD; *P<0.05; n=6 per group). B, Western blot of hepatic SR-B1 from wild-type and ApoE-deficient mice, with actin used as a loading control.

Figure 3. High-density lipoprotein (HDL) turnover in ApoAI transgenic mice liver. A, Time course of 2H incorporation into HDL cholesterol (HDLc). B, Time course of 2H incorporation into human (triangular symbols) and mouse (square symbols) ApoAI. To account for any variations in the total body water labeling, the net labeling of ApoAI at each time point was normalized to water labeling. Insets give the corresponding levels, fractional catabolic rate (FCR), and production rate (PR) for HDLc and human and mouse ApoAI (mean±SD; n=6 per group).
FCR (3.2±0.2%/pool versus 2.5±0.3%/pool; P=0.01) and PR (0.8±0.1 versus 0.6±0.1 mg/kg per hour; P=0.01) were significantly increased in response to myriocin treatment (Figure 4A; Table 2). Myriocin significantly increased both plasma levels (160.8±9.2 versus 198.9±15.1 mg/dL; P=0.01) and PR (3.7±0.3 versus 4.4±0.2; P=0.01) of ApoAI without any effect on FCR (Figure 4B; Table 2).

Myriocin also significantly increased macrophage-specific RCT to plasma at 24 and 48 hours (P<0.05; Figure 5A); however, this treatment did not affect hepatic uptake of cholesterol (Figure VIII in the online-only Data Supplement). The most pronounced effect of myriocin was on RCT to the fecal compartment, which was significantly increased in the myriocin-treated group at each daily time point, leading to a 1.61-fold increase in fecal RCT cumulatively during the 3-day time course (P<0.001; Figure 5B). Thus, myriocin caused higher mobilization of macrophage cholesterol into plasma coupled with increased excretion of sterols to feces. Fecal sterol composition analysis revealed that myriocin led to an ~3-fold increase in excreted [1H]neutral sterols (Figure 5C) without altering [1H]bile acid excretion (Figure 5D). Increased fecal excretion of sterols also was associated with 2-fold induction of hepatic mRNA levels of ATP-binding cassette transporters (Abc) Abcg5 and Abcg8, the half-transporters involved in hepatobiliary elimination of cholesterol (Figure 6). In contrast, myriocin did not have any significant effect on hepatic gene expression of biliary phospholipid transporter Abcb4 (same as Mdr2).

We also assessed the effect of myriocin on hepatic cholesterol metabolism. Hepatic total cholesterol pool did not change because of myriocin treatment (Figure 7A). However, myriocin significantly increased FCR of hepatic cholesterol (1.2%/h versus 0.7%/h; P<0.001; Figure 7B) and cholesterol PR almost 2-fold (Figure 7B) and cholesterol levels. Measured ApoAI turnover rates were within the range of the values reported in the literature using different methods and consistent with the expectations based on genetic modifications in these animals.11,15,16 For example, the human ApoAI FCR we measured in ApoAI transgenic mice of 11.1±0.6%/h agrees well with the human ApoAI FCR values of 11±1%/h obtained in similar mice by radioiodination and after turnover after IV injection.10 In addition, the mouse ApoAI FCR of 3.3±0.4%/h we determined is similar to the previously measured mouse ApoAI FCR of 4.3%/h that was assessed after injection of radioiodinated mouse ApoAI.14 We also demonstrated that myriocin, a drug known to diminish atherosclerosis in mice, stimulated HDL turnover that was associated with increased macrophage-to-feces RCT. Thus, as a simple and safe approach, the 3H2O-based HDL turnover method could be widely applied to study HDL turnover and give insights into HDL functionality in RCT.

Several in vivo and in vitro labeling approaches using radioactive and stable isotopes have been developed to assess HDL turnover and RCT.12,37 Because of safety concerns, radioactive methods are now largely limited to animal studies. Previous human studies of HDL turnover with stable isotopes relied on precursor–product relationships and involve administration of 2 different tracers, that is, a labeled amino acid and acetate or cholesterol. In addition to the inconvenience related to long-term oral consumption or intravenous infusion of tracers and the difficulties in determining the intracellular precursor enrichment, these methods require specialized gas chromatography–combustion–isotope ratio mass spectrometry for the measurement of the low isotopic enrichment of the product.12,38 In this study, we used a single 3H2O tracer and a simple protocol to measure both HDLc and ApoAI turnover in ApoB-depleted plasma. In contrast to other tracers, 3H2O rapidly equilibrates with total body water, intracellular amino acids, and lipogenic substrates (acyetyl-CoA and NADPH), thus eliminating the need for assessment of the true intracellular precursor enrichment, a critical step in a tracer study.

Table 2. Effect of Myriocin on HDL Turnover (n=5)

<table>
<thead>
<tr>
<th>Group</th>
<th>Total Cholesterol</th>
<th>HDL Cholesterol</th>
<th>ApoAI</th>
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<tbody>
<tr>
<td></td>
<td>Levels, mg/dL</td>
<td>Levels, mg/dL</td>
<td>FCR, %/h</td>
</tr>
<tr>
<td>Control</td>
<td>84.6±7.3</td>
<td>48.2±9.1</td>
<td>2.5±0.3</td>
</tr>
<tr>
<td>Myriocin</td>
<td>98.3±17.5</td>
<td>55.4±2.2</td>
<td>3.2±0.2</td>
</tr>
<tr>
<td>P value</td>
<td>NS</td>
<td>NS</td>
<td>0.01</td>
</tr>
</tbody>
</table>

FCR indicates fractional catabolic rates; HDL, high-density lipoprotein; and PR, production rates.
In addition, incorporation of multiple copies of \(^2\text{H}\) results in amplification of isotopic enrichment in ApoAI and HDLc, enhancing measurement sensitivity during the 3- to 7-day labeling experiments we performed in mice. Serial blood sampling allowed us to measure HDL kinetics within each mouse. This protocol also allowed us to combine the measurement of HDL turnover with the macrophage-specific RCT in the same experiment. Furthermore, as a safe, nonradioactive tracer, \(^2\text{H}_2\text{O}\) can be administered in drinking water to free living organisms making this method easily adaptable for use in human studies. Based on previous studies with \(^2\text{H}_2\text{O}\),\(^{21}\) we envision that 0.5% \(^2\text{H}_2\text{O}\) enrichment of total body water for 15 days in humans would allow accurate quantification of both HDLc and ApoAI turnover. It is relatively inexpensive ($\approx$500–$1000/person) compared with traditional cholesterol, acetate, and amino acid tracers ($\approx$3000–$5000/person). The price of the \(^2\text{H}_2\text{O}\)-metabolic labeling experiment in mice is mainly associated with the analyses, as the cost of the \(^2\text{H}_2\text{O}\) tracer per mouse is $<10$.

It is important to note that the exponential rise modeling used in this \(^2\text{H}_2\text{O}\)-metabolic labeling study requires several days for accurate projection of a plateau enrichment of a product. However, it will not be practical to apply this approach to other proteins and lipids with slower turnover rates or when accurate projection to plateau is impossible. In addition, the exponential rise modeling requires serial sampling for accurate fitting. An alternative approach would be a short-term experiment that would require analyses of only 2 samples, that is, the baseline sample before \(^2\text{H}_2\text{O}\) administration and another sample collected during the semilinear rise of the product enrichment. In this case, the FCT can be calculated using precursor/product relationship. These short-term experiments would necessitate the knowledge of the theoretical plateau labeling of the product based on body water enrichment and the number of labeled C-H sites in the product.\(^{26}\)

We applied the \(^2\text{H}_2\text{O}\)-metabolic labeling approach for the assessment of HDL turnover in wild-type and ApoE\(^{-/-}\) mice. Mice lacking ApoE are characterized with reduced plasma HDLc and ApoAI levels, which spontaneously develop atherosclerosis in response to their high levels of non-HDLc.\(^{32}\) The kinetic analysis revealed that HDLc and ApoAI FCR was higher, and their PR lower in ApoE\(^{-/-}\) mice compared with wild-type controls. The discrepancy between mRNA and ApoAI levels demonstrated in this study highlights the importance of protein kinetics measurements. The increased HDLc FCR in ApoE\(^{-/-}\) mice was accompanied by increased hepatic expression of SR-B1, the receptor for HDLc uptake by the liver. The increased SR-B1 expression in ApoE\(^{-/-}\) mice could be one of the potential mechanisms of accelerated HDL turnover. Although the

Figure 4. Effect of myriocin on high-density lipoprotein (HDL) turnover in wild-type mice. Time course of \(^2\text{H}\) incorporation into HDL cholesterol (HDLc; A) and ApoA1 (B) for control (square symbols) and myriocin-treated (triangular symbols) mice (mean±SD; n=5 control group and n=4 myriocin group).

Figure 5. Effect of myriocin on macrophage-specific reverse cholesterol transport (RCT). A, Time course of RCT to the plasma (% of injected \([\text{H}]\)cholesterol) after subcutaneous injection of cholesterol-labeled macrophages. B, Daily and cumulative fecal RCT. C, Fecal \([\text{H}]\) dpm in neutral sterols. D, Fecal \([\text{H}]\) dpm in bile acids. For all panels, open symbols and bars represent control group, and closed symbols and bars represent myriocin group, mean±SD, n=5 control group and n=3 myriocin group (1 mouse was eliminated from myriocin group because of loss of labeled donor foam cells); *P<0.05; **P<0.01; ***P<0.001.
increased HDL turnover and SR-B1 expression in ApoE\(^{-/-}\) mice suggest higher hepatic HDLc uptake, further studies are warranted to determine whether HDL turnover method could be used to measure selective uptake of HDL cholesteryl ester.

Previously, Arai et al\(^{39}\) measured cholesteryl-ether and radio-iodated HDL clearance in control and ApoE-deficient mice. They also observed increased hepatic SR-B1 expression in ApoE-deficient mice; however, the clearance of \(^{1}H\)cholesterol-ether HDL from the plasma was slower in these ApoE-deficient mice.\(^{39}\) The \(^{2}\text{H}_{2}\text{O}\)-labeling method we used labels all pools of HDLc and does so endogenously without disturbing HDL structure or function. However, the radioactive tracer method–used ex vivo labeling of HDL can have profound effects on HDL structure and function. Thus, the current method of \(^{2}\text{H}\)-metabolic labeling to measure HDL kinetics may be more accurate because it may not disturb endogenous HDL function.

In contrast to ApoE-deficient mice, ApoAI transgenic mice with overexpression of human ApoAI have high plasma HDLc and ApoAI, and are protected against atherosclerosis.\(^{40}\) We demonstrated that HDLc PR and ApoAI FCR and PR were increased in ApoAI transgenic mice.\(^{2}\) Presumably, the larger pool of HDLc and ApoAI observed in these animals drives HDL-mediated net cholesterol removal, which is consistent with previous macrophage-specific RCT studies.\(^{41}\) In addition, our results demonstrate a clear relationship between HDL levels and turnover. Combining the data in Tables 1 and 2, we observed that ApoAI levels were directly related to its PR (\(r^{2}=0.9; \ P=0.03\); Figure 8D). Similar, but not significant, association was observed between HDLc levels and PR (\(r^{2}=0.9; \ P=0.06\); Figure 8B). However, there were no consistent inverse correlations between both HDLc and ApoAI levels, and their FCRs (Figure 8A and 8C). Yet, these findings are specific to the models under study, and it is possible that other genetic and environmental factors modulating ApoAI or HDLc levels could act primarily through FCR. For example, in ApoAI turnover studies in normolipidemic humans, ApoAI and HDLc levels were associated with ApoAI PR, and not FCR.\(^{42}\) However, in studies combining normolipidemic and hyperlipidemic humans, HDLc was associated with HDL size and ApoAI FCR.\(^{43}\)

A somewhat similar HDLc turnover study was performed in mice by Osono et al\(^{44}\); however, this study did not detect a relationship between HDLc and centripetal cholesterol flux. However, there are some key design differences between their study and ours. For example, they typically administered \(^{1}H\)-water and determined cholesterol turnover during \(\approx 1\) hour, limiting their findings to the early rapid label incorporation phase, whereas in our studies the measured changes in cholesterol turnover reflect the integration of events during several days. In addition, their study used a cholesterol ester transfer protein transgene to modulate mouse HDL, whereas we used an ApoAI transgene. We conclude that the \(^{2}\text{H}_{2}\text{O}\)-based turnover method described here extends the classical studies of Turley et al\(^{45}\) and Dietschy et al\(^{46}\) thereby allowing one to simultaneously examine lipid and protein kinetics, which could presumably yield novel insight about lipid trafficking in the context of modulating atherosclerosis.

Traditionally, plasma level of HDLc has been considered as a marker of the RCT. As it has been shown by Blum et al\(^{47}\) HDLc (and ApoAI) levels are not quantitatively important determinants of the mass of slowly exchanging body cholesterol pool. This finding disproves the hypothesis that high levels of plasma HDLc are associated with reduced cholesterol in peripheral organs. RCT is a complex process involving ABCA1 and ABCG1-mediated efflux of free cholesterol from extrahepatic tissues to HDL, lecithin:cholesterol acyltransferase and cholesterol ester transfer protein catalyzed HDL remodeling, SR-B1–dependent selective uptake of HDLc by the liver and ABCG5/ABCG8 facilitated excretion into feces.\(^{5}\) Activation or suppression of each of these steps may have concordant or discordant effects on HDLc levels and RCT. For example, hepatic overexpression of SR-B1 in mice reduces atherosclerosis, which is also associated with increased RCT despite the fact that HDLc levels were reduced.\(^{41}\) Thus, HDLc levels are not always predictive of HDL functionality because the static measure of the HDL pool does not reflect the flow of cholesterol through RCT. In this study, we tested the relationship between global flux of HDL and RCT through direct comparison of these 2 methods in same animals. In these experiments, we used myriocin as a pharmacological tool to modulate sphingolipid and HDL metabolism. It is known that oral administration of myriocin protects against atherosclerosis in ApoE\(^{-/-}\) mice, and several
activities of myriocin, such as impaired cholesterol absorption, induced hepatic ApoAI mRNA, and reduced hepatic mRNAs for cholesterol biosynthetic enzymes, may play a role in the protective effect of myriocin against atherosclerosis, 30,48,49 In our study, performed in wild-type mice, myriocin treatment improved macrophage-specific RCT to plasma and feces; however, it did not affect hepatic uptake of cholesterol. It is known that cholesterol excretion is closely associated with phospholipid secretion into the bile and the deficiency of ATP-dependent translocation of phospholipids in Mdr2−/− mice results in impaired cholesterol secretion.50 Interestingly, increased cholesterol excretion attributable to myriocin treatment in this study was associated with increased hepatic gene expression of the half-transporters Abcg5/Abcg8, but not Mdr2 (Abcb4). Abcb4 is involved in phospholipid secretion, and it is coupled to cholesterol excretion. These results suggest dissociation of cholesterol and phospholipid secretion attributable to myriocin treatment. Although the mechanisms of this uncoupling are not clear, similar Mdr2-independent cholesterol secretion has been reported previously.51

Higher mobilization of macropheage cholesterol into plasma coupled with increased hepatic excretion of neutral sterols into feces and reduced cholesterol reabsorption could explain our observed increased in fecal RCT with no changes in hepatic cholesterol uptake and levels. Our study expands the information about the beneficial activities of myriocin to include increased HDL turnover and macrophage-specific RCT, and demonstrates that increased ApoAI levels were associated with increased ApoAI production. Overall, these results demonstrate that HDL turnover method could be used to assess the flow of HDLc, the dynamic HDL function. In addition, the HDL turnover method is compatible with the measures of macrophage-specific RCT. However, in contrast to RCT assay that measures macrophage-specific HDL flux, the HDL turnover method presented in this study estimates the turnover rates of total HDLc and cannot discriminate specific fluxes from different organs, including macrophages. In our study, the labeling of cholesterol in key peripheral organs has not been measured; therefore, we could not evaluate the role of HDL in removal of the specific pool of peripheral cholesterol. As it has been shown by Schwartz et al.,52 ≈80% of esterified cholesterol in HDL originates from low-density lipoprotein, suggesting their hepatic origin. This also is in agreement with our findings on hepatic cholesterol metabolism in myriocin study, which demonstrates that the terminal labeling of hepatic cholesterol in this study is very similar to the labeling of HDLc (data are not presented), suggesting that HDL flux closely associated with the hepatic cholesterol metabolism. However, the future studies are warranted to determine the contribution of different organs to HDLc flux. Because the influx of cholesterol from macrophages into the plasma is very small compared with the flux from the liver, it is less likely that the HDL turnover method could detect the changes in HDLc turnover attributable to macrophage-specific RCT. However, it is noteworthy that the HDL turnover and RCT assays can be performed simultaneously, as it was demonstrated in this study. In some circumstances, the data will be aligned in these 2 assays and complement each other.

Another limitation of this study is that HDLc turnover analysis is based on total cholesterol, that is, free cholesterol plus esterified cholesterol. This was attributable to restricted plasma (25 μL) samples availability from a mouse at each time point. A separate analysis of free and cholesteryl esters is feasible in larger animals and humans with multiple ≈50 to 100 μL plasma collection. Distinction of free and esterified cholesterol in HDL in combination of cholesterol turnover analyses in low-density lipoprotein and very low-density lipoprotein particles, and multicompartmental data analysis would yield useful information on the activities of cholesterol ester transfer protein, HDL remodeling, and cholesterol/cholesterol ester exchange between different particles.

Recently, a newly developed assay was described that uses a cholesterol stable isotope dilution method to assess total RCT in humans in vivo.76 This method requires extensive (≈24–32 hours) and expensive [2,3-13C]-cholesterol infusion, followed by multicompartmental modeling to measure tissue cholesterol efflux and plasma cholesterol esterification rates.
The nonabsorbable tracer, 3H2-sitastanol, is also required for assessment of stool recovery for measuring total fecal sterol excretion. This approach assumes the complete equilibrium of the infused tracer with hepatic cholesterol. As discussed previously, it is difficult to prove this assumption in humans.53 In addition, this method estimates peripheral cholesterol efflux based on the rate of label appearance in total plasma cholesterol and cholesterol esters without distinguishing among the various lipoprotein classes. In contrast, the HDL turnover method presented in this study makes no assumptions about tracer equilibrium because 3H2O, an inexpensive tracer, equilibrates rapidly with total body water and the precursors of lipid and protein biosynthesis.24,54 In addition, our method specifically measures the flux of cholesterol in the HDL compartment, which may be a useful measure of dynamic HDL function relevant to its role in RCT.

In conclusion, we have developed a safe and simple stable isotope–based method for estimating HDL turnover that is compatible with the radioactive macrophage–specific RCT assay. Oral 3H2O administration has been previously used in human studies to measure gluconeogenesis, protein synthesis, and lipogenesis.21,23,25,35 Thus, we propose that our method can be easily adapted to measure HDL turnover in humans, and be used to study genetic, environmental, and pharmacological effects on HDL turnover and its implications for HDL function, RCT, and atherosclerosis.

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Disclosures
Dr Hazen reports being listed as coinventor on pending and issued patents held by the Cleveland Clinic relating to cardiovascular diagnostics and therapeutics. Dr Hazen reports having been paid as a consultant or speaker for the following companies: Abbott Diagnostics, Cleveland Heart Laboratory, Esperion, Lilly, Liposcience Inc, Merck & Co, Inc, and Pfizer Inc. Dr Hazen reports receiving research funds from Abbott, Cleveland Heart Laboratory, Liposcience Inc, and Pfizer Inc. Dr Hazen reports having the right to receive royalty payments for inventions or discoveries related to cardiovascular diagnostics or therapeutics from Cleveland Heart Laboratory and Esperion.

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Takhar Kasumov, Belinda Willard, Ling Li, Min Li, Heather Conger, Jennifer A. Buffa, Stephen Previs, Arthur McCullough, Stanley L. Hazen and Jonathan D. Smith

2H2O-Based High-Density Lipoprotein Turnover Method for the Assessment of Dynamic High-Density Lipoprotein Function in Mice


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Supplement Material

$^{2}$H$_2$O-Based HDL Turnover Method for the Assessment of Dynamic HDL Function

By: Takhar Kasumov, Belinda Willard, Ling Li, Min Li, Heather Conger, Jennifer A. Buffa, Stephen Previs, Arthur McCullough, Stanley L. Hazen, Jonathan D. Smith
Supplemental Figure I. Testing of post-secretion labeling of ApoAI due to hydrogen/deuterium exchange with $^2$H$_2$O. ApoB-depleted HDL sample was incubated with 10% $^2$H$_2$O at room temperature for 7 days. This sample in parallel with the control (untreated) HDL sample was processed and analyzed by LC-MS. Black and red lines present the isotopomer distribution of control and $^2$H$_2$O-treated samples, respectively. Quantification of net labeling using equation 4 demonstrates that no measurable $^2$H-labeling was detected due to hydrogen/deuterium exchange with $^2$H$_2$O.
Supplemental Figure II. $^2$H-labeling and relative plasma levels of HDLc (A) and ApoAI (B) analyzed by mass spectrometry. ApoB100-depleted plasma samples from wild type mice at the baseline and after 1 day of $^2$H$_2$O labeling experiment were spiked with equal amounts of $[^2$H$_6$]-cholesterol and VAPL($^{13}$C$_6$)GAEL($^{13}$C$_6$)QESAR peptide (internal standards). HDLc was analyzed by GC/MS and tryptic digested ApoAI peptides were analyzed by high resolution LC/MS. After one day of $^2$H$_2$O labeling, the intensities of heavy isotopomers ($M_1$-$M_4$) were increased relative to monoisotopic peak ($M_0$). Areas under curves of each isotopomer of cholesterol and ApoAI derived peptide VAPLGAELQESAR were measured for quantification of their $^2$H-enrichments. The integrated peak areas ratios HDLc/$[^2$H$_6$]-cholesterol and VAPLGAELQESAR/VAPL($^{13}$C$_6$)GAEL($^{13}$C$_6$)QESAR were used for calculation of concentration of HDLc and ApoAI, respectively.
Supplemental Figure III. HDL turnover measured in a wild type mouse assessed with $^2$H$_2$O-metabolic labeling technique. A: Time course of $^2$H incorporation into HDLc. B and C: Time course of $^2$H incorporation into ApoAI assessed using 3 different peptides. Because of distinct sequences each analyzed ApoAI peptide reached different asymptotic labeling (Panel B). Normalization of the labeling at all time points for the maximum plateau labeling of each analyzed peptide allows one to see that all 3 peptides overlay (Panel C). The FCR and half-life of HDLc and ApoAI were determined by fitting the time course labeling into first order kinetic curves.
**Supplemental Figure IV.** Effect of ApoE deletion (A) and human ApoAI over expression (B) on hepatic murine ApoAI mRNA expression (means ± SD; N=6 per group *p<0.001.)
Supplemental Figure V. Time course labeling of body water in ApoAI transgenic mice.
Supplemental Figure VI. ApoAl turnover in Apol transgenic mice. Normalization of the labeling at all-time points for the maximum plateau labeling of mouse and human ApoAl peptides illustrates differences in the turnover rates of mouse and human protein.
**Supplemental Figure VII.** Time course labeling of total body water in wild type mice.
Supplemental Figure VIII. Macrophage-specific RCT to the liver. Myriocin treatment did not affect hepatic uptake of cholesterol.
MATERIALS AND METHODS

Materials.
HPLC grade solvents for nanospray chromatography and sample preparation were purchased from Fluka (Milwaukee, MO). Pure standards of [2H6]cholesterol and N-(9-fluoronymethyloxy-carbonyl-[13C6]-leucine (L-[13C6]-Fmoc-leucine), were purchased from (Cambridge Isotope Laboratories, Andover, MA, purity > 98%). All other chemicals, including myriocin were from Sigma-Aldrich (St. Louis, MO). Myriocin (2.2 mg myriocin/kg diet) in chow diet was prepared by Teklad Diets (Harlan Laboratories, Madison, WI).

The labeled peptide VAPL(13C6)GAEL(13C6)QESAR was synthesized using a solid-phase method in the Cleveland Clinic Molecular Biotechnology Core on a 396 52 Peptide Synthesizer (Advanced Chem Tech, Louisville, Kentucky). Stable isotope labeled L-[13C6]-Fmoc-leucine was coupled in the peptide sequence to give a molecular mass shift of 12 Da from the unlabeled endogenous peptide. The molecular weight of the purified peptide was verified by ESI-MS and was found to yield M+2 ion with the expected 676.88 average mass and an isotopic purity of 98%. The stock solution of labeled peptide VAPL(6C13)GAEL(6C13)QESAR was made at a concentration of 30 μmol/L in acidic water (pH = 3). This solution was divided into 0.05 ml fractions and stored at -80ºC until usage.

Animal studies.
All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the Cleveland Clinic and were performed in accordance with NIH guidelines. Female C57BL/6 mice (~25g) were purchased from Charles River Laboratories (Wilmington, MA). ApoE−/− and homozygous ApoAI transgenic mice were originally purchased from JAX (Bar Harbor, ME) and bred in our animal care facility. All animals were housed in our animal care facility with a 12:12 h light:dark cycle. The animals had free access to chow diet (20% kcal from protein, 70% kcal from carbohydrate and 10% kcal from fat, Harlan Teklad) and water.

HDL turnover studies in ApoE−/− and ApoAI transgenic mice.
Two days after taking baseline blood samples through bleeding from the lateral saphenous vein, eight week old ApoE−/− mice (n=6) and age and body weight matched wild type mice received a loading dose of 2H2O saline solution (22 µl/g body weight by intraperitoneal injection) and were given drinking water enriched with 2H2O (6%). Saphenous vein blood samples (~80 µl) were collected at 4, 10 and 24 hrs. Animals were maintained on 6% 2H2O for six days and were sacrificed after obtaining a terminal blood sample (~1ml) by cardiac puncture. Blood samples were centrifuged (2000 g for 10 minutes) immediately. Plasma was used for the analysis of 2H-labeling of the total body water (5 µl) and for isolation of the HDLc fraction (25 µl), as describe below. After perfusing the heart with cold PBS, the livers were frozen at -80ºC for later analysis.

In a separate study human ApoAI transgenic mice (n=6) were treated with the same dose of 2H2O. In addition to saphenous vein baseline blood samples, blood samples were collected at 4, 8, 24 and 72 hrs of 2H2O administration. At the seventh day of labeling experiment animals were anesthetized and terminal blood samples were collected through cardiac puncture. Blood samples were centrifuged immediately and plasma samples were processed for isolation of HDL.

HDL turnover and macrophage-specific RCT in wild type mice: Effect of myriocin.
2H2O-metabolic labeling and the macrophage-specific RCT experiments were conducted simultaneously in C57BL/6 mice fed chow diet with or without myriocin (2.2 mg/kg diet). Myriocin is a specific inhibitor of serine palmytoyltransferase (SPT)-the rate limiting enzyme in sphingolipid biosynthesis. Previously it has been shown that oral administration of myriocin for 12 weeks decreases plasma sphingolipids and reduces atherosclerosis in ApoE−/− mice. In this experiment we tested whether myriocin consumption for 2 weeks affected macrophage-specific
RCT and HDL turnover. For direct comparison of HDL turnover with RCT method\textsuperscript{2}, we reduced the dose of \textsuperscript{2}H\textsubscript{2}O (15 \mu l of 99\% atom percent enrichment \textsuperscript{2}H\textsubscript{2}O-labeled saline per g body weight) and completed the study in 3 days. Two days after the baseline blood sampling, 5 male, 8-10-week-old mice from each group were caged individually with free access to food and water. Bone marrow macrophages were cultured from wild type mice for 14 days in DMEM supplemented with 20\% L-cell conditioned medium (as a source of macrophage colony-stimulating factor) and 10\% fetal bovine serum. To load and label macrophages with \([\textsuperscript{3}H]\)cholesterol, cells were incubated for two days with DMEM containing 20\% L-cell conditioned medium, and acetylated LDL (50 \mu g/ml), which was pre-incubated with \([\textsuperscript{3}H]\)cholesterol (Perkin-Elmer) for 30 min at 37\textdegree C in order to achieve a final concentration of 2 \mu Ci/mL in the labeling media. Cells were washed twice with DMEM and these bone marrow macrophages (4-5\times10\textsuperscript{6} cells containing 5\times10\textsuperscript{6} dpm \([\textsuperscript{3}H]\)cholesterol in 0.5 ml of minimum essential medium) and \textsuperscript{2}H\textsubscript{2}O saline were subcutaneously injected simultaneously to measure macrophage-specific RCT and turnover of HDL\textsubscript{c} and ApoAI, respectively. Animals were returned to their cages and their drinking water was replaced with water enriched with 5\% \textsuperscript{2}H\textsubscript{2}O. Retroorbital blood samples (~80 \mu l) were collected at 4, 8, 24 and 48 hours under isoflurane anesthesia. After 72 hours, the mice were sacrificed and terminal blood was collected. After perfusing the heart with cold PBS, the liver was weighed, frozen, and saved. Plasma samples were isolated immediately and were divided into two parts. One part (10 \mu l) of plasma was analyzed for \([\textsuperscript{3}H]\)cholesterol radioactivity using liquid scintillation counting. The second part was used for the analysis of \textsuperscript{2}H-labeling of plasma (5 \mu l) and HDL\textsubscript{c} isolation (25 \mu l). Feces were collected daily during the entire experiment. Hepatic and dried fecal homogenates were subjected to scintillation counting as previously described\textsuperscript{3} and RCT to the plasma, liver, and fecal compartments was calculated as the \% of the injected \([\textsuperscript{3}H]\)cholesterol dosage. The fecal homogenate was separated into neutral sterol and bile acid fractions as described\textsuperscript{3} in order to determine the \% of \([\textsuperscript{3}H]\)dpm in these fractions. The \textsuperscript{2}H-labeling of plasma, HDL\textsubscript{c} and ApoAI were analyzed as described below.

Analytical

Gene Expression Analyses

Total RNA was isolated from the mouse liver tissue using RNase Mini Kit (Qiagen Inc. Valencia, CA). One \textgreek{g} of total RNA was reverse transcribed using the Advantage\textsuperscript{R} RT-for-PCR Kit (Clontech Laboratories, Inc. Mountain View, CA) with random decamers as primer. Real-time PCR amplification was performed using Brilliant SYBR Green QPCR Master Mix (Agilent Technologies, Inc. Santa Clara CA) and gene-specific primers in an Mx3000p PCR machine (Agilent Technologies, Inc. Santa Clara CA) in duplicate. The relative amount of target mRNA was determined using the cycle threshold (Ct) method by normalizing target mRNA Ct values to that of \textgreek{b}-actin. Fold induction ratios were calculated relative to basal conditions for each genotype using the formula: 2\textsuperscript{ΔΔCt}. The primer sequences are as follows: \textgreek{b}-actin forward: 5\textsuperscript{′} - CCTTCTGGGCATGGAGTCT-3\textsuperscript{′}, reverse: 5\textsuperscript{′} - GGAGCA ATGATCTTG ATCTTC-3\textsuperscript{′}; SR-B1 forward: 5\textsuperscript{′} - ATGGGCCAGCGTGCTTTTATGAAC-3\textsuperscript{′}, reverse: 5\textsuperscript{′} - ACCGCCCGTAGACAGTGAAGACC-3\textsuperscript{′}; ApoAI forward: 5\textsuperscript{′} - ACGTATGGCAAGATCACTCT-3\textsuperscript{′}, reverse: 5\textsuperscript{′} - AGAGCTCCACATCCTCTTTCC -3\textsuperscript{′}; ABCG5 forward: 5\textsuperscript{′} - TGGCCCTGCTCAGCATCT-3\textsuperscript{′}, reverse: 5\textsuperscript{′} - ATTTTTAAAGGA ATGGGCATCTCTT-3\textsuperscript{′}; ABCG8 forward: 5\textsuperscript{′} - CCGTCGTCA GATTTC CAA TGA -3\textsuperscript{′}, reverse: 5\textsuperscript{′} - GGCTTCCGACCCATGAATG -3\textsuperscript{′}; ABCB4 forward: 5\textsuperscript{′} - AACACAGCCAACCTTGGAAC -3\textsuperscript{′}, reverse: 5\textsuperscript{′} - TGTTGCAATCTTTCCAGCAG-3\textsuperscript{′}. All primers used for real-time PCR analysis were synthesized by Integrated DNA Technologies, Inc (Coralville, NJ). Immunoblot analysis of mouse SR-B1 was carried out with liver proteins solublized in RIPA buffer containing protease inhibitors cocktail. Ten \mu g of reduced total protein samples were run on pre-cast SDS-PAGE gel (4-20\% acrylamide) and then transferred to PVDF membrane. Mouse SR-B1 was detected by rabbit anti-mouse SR-B1 after blocking with 5\% milk in TBST. The primary antibody was
detected using horse radish peroxidase (HRP) conjugated secondary antibody with 1: 4000 dilution in TBST followed by ECL Western blotting detection reagents.

**Total body water enrichment**

$^2$H-enrichment of total body water was measured using a modification of the acetone exchange method. Briefly, 5 µl of plasma was incubated with 2 µl of 10 M KOH, and 5 µl of pure acetone in a 2 ml glass screw-cap GC vial at room temperature for 4 hours. One µl of acetone vapor from the headspace was directly injected for gas-chromatography mass spectrometry (GC-MS) analysis. The enrichment of acetone was determined using electron impact ionization and selected ion monitoring. Acetone was monitored at ions m/z 58 (M₀), 59 (M₁) and 60 (M₂). The biological samples were analyzed in parallel with the calibration curve samples containing from 0 to 5% $^2$H₂O. The regression equation of the calibration curve was used for the calculation of $^2$H₂O enrichment in biological samples.

**Hepatic cholesterol analysis**

Liver samples (~30-40 mg) from $^2$H₂O treated and untreated wild type control mice in parallel with the calibration curve samples were spiked with 50 µL of 1 mM [²H₆]-cholesterol, homogenized in 0.5 ml of 1N NaCl and extracted by the Bligh-Dyer method. After the evaporation of the solvents, the lipids were saponified with 1N KOH/70% ethanol (v/v) for 2 h at 70 °C vortexing occasionally. Samples were then evaporated to dryness and suspended in 150 µl of 1N HCl. Cholesterol was extracted with 1.0 ml of pentane. The solvent was evaporated and the dried residue was derivatized with 65 µl of bis(trimethylsilyl) trifluoroacetamide + 1% trimethylchlorosilane (TMS) at 70 °C for 45 min. The $^2$H enrichment and concentration of cholesterol was determined using an Agilent 5973N-MSD equipped with Agilent 6890 GC system. Electron impact ionization (70 eV) with selected ion monitoring (SIM) of m/z 368-371 (M₀-M₅, endogenous cholesterol) and 374 (M₆, [²H₆]-cholesterol internal standard) dwell time of 10 ms per ion was used for all analyses. Hepatic cholesterol content (nmol/gram wet weight) was then quantified using a calibration curve and the ratios of the integrated peak areas.

**HDLc analysis**

Plasma samples (25 µL) were placed in a 7 x 20 mm thick-wall polycarbonate centrifuge tube (Beckman Coulter, CA). One volume of PBS was slowly added on top of each plasma sample and then the samples were centrifuged at 21300 g for 3 hrs at 4 °C to spin up VLDL. The lower phase (25 µL) of solution was removed and any ApoB100 containing particles (LDL and LDL) where precipitated with 3 µL of a magnesium chloride/dextran sulfate reagent (Stanbio Laboratory, Boerne, TX). After mixing and incubating at room temperature for 5 minutes, the supernatant containing the ApoB-depleted plasma was recovered and used for the analysis of both HDLc and ApoAI. HDLc and ApoAI levels were quantified using the isotope dilution method by mass spectrometry. Briefly, the ApoB-depleted plasma samples were spiked with 50 µL of 1 mM [²H₆]-cholesterol solution in chloroform and 50 µL of 30 µM synthetic peptide VAPL($^6$C₁₃)GAEL($^6$C₁₃)QESAR solution in water. HDL proteins, including ApoAI and synthetic peptide (internal standard) were precipitated with 1 mL of cold acetone (-20 °C). Samples were incubated at -20 °C for 4 hours and then they were centrifuged at 2000 g for 5 minutes. The pellets were saved for the analysis of ApoAI. The supernatant was used for the analysis of HDLc. After the evaporation of acetone total HDLc was analyzed by the GC-MS as described above.

**Sample preparation for ApoAI analysis**

The pellets isolated from ApoB100 depleted plasma were washed with 600 µL of cold acetone (-20 °C) and centrifuged at 2000 g for 5 minutes. The supernatants were discarded and the pellets were air dried. Proteins were denaturated by an addition of 100 µL of 6 M urea solution.
in 100 mM Tris buffer (pH 8). After mixing, the samples were incubated at 4 °C overnight. Two μL of the solution was diluted with 10 μL of 100 mM Tris buffer (pH 8). To reduce the thiol groups of proteins, the samples were reacted with dithiothreitol (DTT) (9 μL, 30 mg/ml in 100 mM pH 8 Tris buffer) for 20 minutes at room temperature. Samples were alkylated with an excess of iodoacetamide (9 μL of 36 mg/ml solution in 100 mM pH 8 Tris buffer) for 20 minutes at room temperature. The excess of iodoacetamide was reacted with DTT. The solution was adjusted to pH 8 by titration with 100 mM ammonium bicarbonate. Proteins were digested in solution with an excess of Promega sequencing grade trypsin (10 μL of 100 ng/μL trypsin solution in 100 mM pH 8 Tris buffer) at room temperature overnight. Samples were desalted through solid phase extraction using a Pierce C18 Pepclean spin column. The peptides were eluted with 2 X 20 μL 70% acetonitrile and the solvent was evaporated in a Speedvac. Samples were reconstituted in 30 μL of 1% acetic acid. Five μL of the sample solution was injected for the LC-MS analysis.

**LC-MS/MS analysis.**

The chromatographic separation of the protein digest was performed by an PROXEON EASY-nLC II HPLC (Thermo Scientific) with a reverse phase nano-column (C18, 75 μm×150 mm, 3 μm, Newobjective) using a mobile phase A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile). A 140-minute stepwise gradient started with 2% of mobile phase B, after 10 minutes of desalting, the mobile phase B was linearly increased to 40% in 120 minutes. Mobile phase B then ramped to 80% in 5 minutes and then held at 80% B for 15 minutes. Subsequently mobile phase B was decreased to 2% in 2 minutes and equilibrated for 10 minutes with 2% of B. Tandem mass spectra were recorded on a hybrid LTQ Orbitrap Velos (Thermo Electron Corp., Bremen, Germany) operated in a positive ion mode using electro spray ionization (ESI). 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 spray voltage of 2.5 kV. The inlet capillary temperature was maintained at 200°C. A data-dependent method was used for data acquisition. Each full MS scan using Orbitrap (high resolution FT instrument) at 60,000 resolution at m/z 400 was followed by 10 collisionally induced dissociation (CID) MS-MS scans on LTQ mass spectrometer.

Isotope incorporation was assessed based on the mass isotopomer distribution analysis of a high resolution full scan spectra recorded in Orbitrap MS. Mass isotopomers are molecules that differ by the presence of different heavy isotopes resulting in a mass spectrum with a baseline monoisotopic (M₀) peak followed by distinct heavy isotopomer (Mᵢ, where i is an integer > 0) peaks. Only ions with good abundance (10⁶-10⁷ intensity) were selected for an accurate quantification of label incorporation and protein concentration. 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±0.05 Da). The kinetics of mouse ApoAI were analyzed using isotopic distribution of VAPLGAELQESAR (analyzed as M₁² ion with the m/z of 670.87) and other tryptic mouse ApoAI peptides. Because of the sequence difference, distinct peptides, including VSFLSALEYTK (M₁² ion with the m/z of 693.86) were analyzed to assess the kinetics of human ApoAI in ApoAI transgenic mice. The plasma levels of mouse ApoAI were quantified using the ratios of the integrated peak areas of tryptic VAPLGAELQESAR and synthetic VAPL(¹³C₅)GAEL(¹³C₅)QESAR (670.87/676.88) peptides from the full scans. Human ApoAI levels in ApoAI transgenic mice were measured by ELISA using a polyclonal goat ant-human ApoAI anti-body.

**Database Searching.**

For the identification of proteins, the MS data were analyzed using all CID spectra collected in the experiment. Peak lists were generated using Thermo Electron Proteome Discoverer V1.3.
software and compiled into Mascot generic format (.mgf). The .mgf files searched against the National Center for Biotechnology Information mouse reference sequence database (ftp://ftp.ncbi.nih.gov/refseq/) released on December 20th, 2011 containing 30,438 entries. The search was performed using carbamidomethyl as a fixed modification of cysteine, oxidation as an optional modification of methionine and allowing one missed cleavage. The mass tolerances for the precursor and product ions were 50 ppm and 1.5 Da, respectively. A score of > 35 was considered as significant. The interpretation process was aided by additional searches using the Blast (http://blast.ncbi.nlm.nih.gov/Blast.cgi) as needed. Mouse and human ApoAI were identified based on multiple unique peptides with 99% confidence and false discovery rate of 1%.

**Calculation of the fractional catabolic rate (FCR).**

We assume that ApoAI and cholesterol levels do not change in the adult mice during the \(^2\)H\(_2\)O-metabolic labeling study period, and that there is steady-state flux of all lipids and proteins, including cholesterol and ApoAI. Thus, at steady state the rate constant represents both the fractional synthesis rate (FSR) and the fractional catabolic rate (FCR). The fractional catabolic rates (FCRs) of ApoAI and HDLc were determined based on a single compartmental model by fitting a time course of M\(_1\) and net labeling of HDLc or a tryptic ApoAI peptide (E (t)), respectively, to an exponential rise curve equation:

\[
E (t) = E_0 * (1-e^{-kt})
\]  

This equation allows determination of asymptotical total labeling (E\(_0\)), the rate constant (k) and the half-life (t\(_{1/2} = \text{ln}2/k\)) of HDLc and tryptic ApoAI peptides. Total labeling was calculated using the formula:

\[
\text{MPE} = \text{MPE}_{M_1} x 1 + \text{MPE}_{M_2} x 2 + \ldots + \text{MPE}_{M_i} x i.
\]  

where MPE\(_{M_i}\) is the molar percent enrichment of an isotopomer and calculated as

\[
\text{MPE of } M_i = \frac{A_{M_i}}{\sum(A_{M_0} + A_{M_1} + \ldots + A_{M_n})}
\]  

where A\(_{M_i}\) represents the area under the curve for \(i^{th}\) isotopomer.

The net labeling (isotopic excess) due to \(^2\)H-incorporation is calculated as the difference of total MPE at each time point and the baseline MPE calculated at t=0:

\[
\text{Net labeling (t)} = \text{MPE (t)} - \text{MPE (0)}
\]

In some instances the FCR and half-life of ApoAI were determined based on the time course labeling of multiple peptides. For this purpose, the labeling of each analyzed unique peptide at all time points was normalized for their maximum plateau labeling and averaged. Thus, aggregation of multiple peptides yields the averaged turnover curve of a protein.

Hepatic fractional catabolic rate of total cholesterol was calculated using the formula:

\[
\text{FCR} = \frac{\text{MPE of Hepatic Cholesterol}}{(N \times \text{MPE of plasma water} \times \text{time})}
\]

where MPE is net labeling of hepatic cholesterol at the 72 h of \(^2\)H\(_2\)O administration and it is calculated using formula (4). N represents the number of exchanged hydrogen atoms and assumed to be 25 for cholesterol.
The production rates (PR) of ApoAI, HDLc and hepatic total cholesterol were calculated as the product of FCR and their respective pool size:

\[
PR (g \times kg^{-1} \times h^{-1}) = \text{pool size} \times \text{FCR}
\] (6)

where the pool size (absolute content) in circulation is the product of HDLc or ApoAI concentrations and plasma volume, estimated as 45 ml/kg body weight. The pool size of hepatic cholesterol (mg/g wet weight) was calculated using the isotope dilution method by mass spectrometry.

**Data Presentation and Statistical Analysis.**

The average of duplicate GC-MS and LC-MS injections, which differed less than 2%, were used for mass-spectrometric analysis. Data are shown for the average of 4-6 mice at each time point unless indicated otherwise. To account for any variations in the total body water labeling between animals, the net labeling at each time point was normalized to water labeling. When multiple peptides were used for ApoAI kinetics analysis, a time course of the normalized $^2$H excess labeling curve was constructed for each peptide. For this purpose measured labeling at each time point was divided by the plateau labeling of a peptide. Error bars in these curves represent standard deviation.

A paired t-test was used to identify differences between ApoE-/- and wild type groups. The statistical significance of differences between the rate constants of ApoAI and cholesterol from control and myriocin treated groups were tested using an unpaired two-tailed t-test. A linear regression analyses was used to assess the relationship between concentration and turnover of HDLc and ApoAI. P< 0.05 considered statistically significant.

**Reference**