A New Labeling Approach Using Stable Isotopes to Study In Vivo Plasma Cholesterol Metabolism in Humans

K. Ouguerram, M. Krempf, C. Maugeais, P. Maugère, D. Darmaun, and T. Magot

A method to study reverse cholesterol transport in humans was developed using stable isotopes and kinetic analysis. Three normolipidemic subjects received simultaneous intravenous infusions of deuterated leucine and ^{13}C-acetate for 14 and 8 hours, respectively. Deuterium enrichment was measured in protein-bound leucine in apolipoproteins (apo) B-100 and A-I (using gas chromatography coupled with mass spectrometry [GCMS]) and ^{13}C-enrichment in unesterified cholesterol and cholesteryl ester (using gas chromatography coupled to isotope ratio mass spectrometry [GC-C-IRMS]) in very–low-density lipoprotein (VLDL), low-density lipoprotein (LDL), and high-density lipoprotein (HDL) during the tracer infusion. Curves were analyzed using multicompartmental analysis. This protocol is suitable to quantify the different processes involved in reverse cholesterol transport (RCT) in humans, including cholesterol esterification, transfer of cholesteryl ester from HDL towards apo B-100–containing lipoproteins, and the contribution of VLDL, LDL, and HDL in the final steps of RCT. In agreement with previous data from kinetic analysis of radiotracer experiments, our results suggest that in fasting normolipidemic subjects the major fraction of cholesteryl ester enters plasma through esterification in HDL (more than 95%). The major fraction of cholesteryl ester disappears through apo B-100–containing lipoproteins (VLDL and LDL) catabolism (mean of 82%) rather than through removal from HDL (mean of 18% with approximately an equal part for apo AI-dependent and independent catabolism, respectively, 7% and 11%). We conclude that this protocol could be applied to study the modulation of these processes by nutrition, diseases, or pharmacologic treatments.

REVERSE CHOLESTEROL TRANSPORT (RCT) allows cholesterol movement from peripheral cells via plasma towards the liver, where cholesterol can be metabolized and cleared from the body as bile acids. RCT is initiated by the efflux of unesterified cholesterol from cells into high-density lipoprotein (HDL containing apolipoprotein [apo] A-I), a step followed by an esterification of cholesterol in these lipoproteins by lecithin cholesterol acyl transferase (LCAT). Transfers towards the liver involve 3 possible mechanisms.[1] They can be performed through HDL catabolism (ie, simultaneous uptake of apo A-I and cholesteryl ester) or via a selective uptake of HDL cholesteryl ester involving the recently identified receptor, SR-BI.[2] This transfer towards the liver can also be performed through cholesteryl ester transfer mediated by cholesteryl ester transfer protein (CETP) towards other lipoproteins, very–low-density lipoproteins (VLDL), and low-density lipoproteins (LDL), containing apo B-100, and followed by an hepatic uptake of these lipoproteins (ie, simultaneous uptake of apo B-100 and cholesteryl ester). The relative importance of these 3 pathways is controversial in humans and probably differs between species and the various physiologic or nutritional situations.[3]

RCT is considered an anti-atherogenic process, and epidemiologic studies have established that the concentration of plasma HDL or HDL cholesterol is inversely correlated with the incidence of cardiovascular disease.[4] However, the atherogeneicity of CETP is controversial,[5,6] as cholesteryl ester transfer activity can provide cholesteryl ester to LDL, a well-known risk factor for coronary heart disease. A safe, ethical method to quantify in vivo in humans the different pathways involved in RCT and discriminate the contribution of HDL catabolism or cholesterol ester transfer from these lipoproteins to apo B-100–containing lipoproteins could therefore be useful.[2]

In vitro methods for CETP activity measurement can estimate this pathway and have been developed in humans.[7] Beyond its technical difficulties, the latter approach, however, only provides an indirect estimate. In vivo protocols with radioisotopes using endogenous[8] or exogenous[9] labeling of cholesterol alone[8] or together with LDL apo B-100[9,10] have been proposed in the past. This approach underlined the role of cholesteryl ester movements in RCT in normolipidemic men and has been successfully applied in various conditions including liver disease[8] or treatment by statins.[9] The use of radioisotopes is, however, questionable for ethical and safety considerations, and studies on apo B-100 and A-I metabolism have been more recently performed in humans with stable isotopes.[11,12] In the field of cholesterol, stable isotopes have been used concerning turnover of whole body cholesterol.[13,14] However, with regard to plasma cholesterol turnover, after pioneering qualitative studies using stable isotopes,[15] the only reported quantitative kinetic data on cholesteryl ester turnover were obtained in an animal model[16] and humans[17] by means of radiotracer methods.

Therefore, we developed a method and a model to access RCT by using an endogenous labeling of plasma lipoproteins with stable isotopes in vivo in humans. With this new method with combined tracer infusion, apo A-I, B-100, free, and esterified cholesterol are simultaneously labeled and analyzed.

MATERIAL AND METHODS

Experimental Protocol

Three adult normolipidemic and normal weight subjects (subjects 1, 2, and 3; 2 men and 1 woman, weighing, respectively, 65, 68, and 56 kg; 25, 27, and 24 years old) were included in this study. They consumed a diet composed of 45% of the usual daily caloric intake as

From the Centre de Recherche en Nutrition Humaine, INSERM U539, Nantes, France.

Submitted July 19, 2000; accepted July 16, 2001.

Address reprint requests to K. Ouguerram, MD, Centre de Recherche en Nutrition Humaine, INSERM U539, CHU Hôtel-Dieu, Nantes, France.

0026-0495/02/5101-0010$35.00/0
doi:10.1053/meta.2002.29006

carbohydrate, 40% as fat, and 15% as protein, for at least 1 week prior to the study. The experimental protocol was approved by the ethical committee of Nantes University Hospital, and written informed consent was obtained before the study was started.

Stable isotope solutions were prepared under sterile conditions by the Hospital Pharmacy (Professor F. Ballereau) using sterile saline, filtered through 0.22-μm filters and stored at 4°C until used. The stable-isotope turnover study was performed under fasting conditions, starting at 8 AM after an overnight fast. The endogenous labeling was performed by administration of L-[5,5,5-$^2H_3$] leucine (99.8 Atom% ^{2}H3; Cambridge Isotope Laboratories, Andover, MA) as endogenous marker of apo A-I and B-100 and of [1-2^{13}C]acetate (99% Atom% enrichment; Tracer Technologies IMC, Sommerville, MA) as an endogenous marker of cholesterol. Tracers were dissolved in a 0.9% saline solution and tested for sterility and absence of pyrogens before the study. Two plastic catheters were placed intravenously (IV) into contralateral arm veins: 1 was used for the tracer infusion, and the other was used for the frequent blood sampling during the study. The subjects received simultaneously a primed-constant infusion of deuterated leucine (10 μmol/kg body weight injected as a bolus IV over 1 minute and then 10 μmol/kg body weight/h during 14 hours) as previously published[18,19] and [1-2^{13}C]acetate (4 μmol/kg body weight injected as a bolus IV over 1 minute and then 4 μmol/kg body weight/h during 8 hours. Venous blood samples were withdrawn in sterile tubes (Venoject, Paris, France) containing EDTA (final concentration of 0.2 mmol/L) and dithiobisnitrobenzoic (DTNB) acid as an inhibitor of LCAT reaction, final concentration 0.2 mmol/L) at different times: 10, 30 minutes, 1 hour, 1.5 hours, 2 hours, and hourly until the end of perfusion. Plasma was immediately separated by centrifugation for 30 minutes at 4°C; sodium azide, an inhibitor of bacterial growth, and Pefabloc SC (Interchim, Montluçon, France), a protease inhibitor, were added to blood samples at a final concentration of 1.5 mmol/L and 0.5 mmol/L, respectively. The plasma was then stored frozen at -20°C for later determination of cholesterol and apo concentration and isotopic enrichments. VLDL, LDL, and HDL were isolated by density gradient ultracentrifugation as previously described.[20] Density gradients, adapted from the method of Terpstra et al,[21] were prepared in 12 mL polyallomer tubes as follows: 1.4 mL distilled water, 1.5 mL saline (density = 1.006), and 1.5 mL (density = 1.019), 2.5 mL (density = 1.063), 3 mL (density = 1.120), and 2 mL serum adjusted to a density of 1.210 by the addition of solid potassium bromide (KBr). The tubes were centrifuged for 24 hours at 15°C (Hitachi RPS 40T rotor, Hialeah, FL) at 40,000 rpm (Himac CP70, Hitachi). The first 22 fractions (0.5 mL each) were collected from the meniscus. Individual samples were pooled into the following 3 classes: VLDL (density $<$ 1.006; fractions 1 to 4), LDL (density between 1.006 and 1.063; fractions 5 to 11), and HDL (density $>$ 1.063; fractions 12 to 22). We assessed the separation densities from a control tube prepared using a saline solution with a density of 1.21 and by measuring the density of the 22 fractions by refractometric analysis after ultracentrifugation under the same conditions. In these conditions, lipoprotein recovery was greater than 85%.[20]

Analytical Methods

Analysis of apo was performed as previously described for B-100[18] and A-I.[19] Briefly, HDL, VLDL, and LDL were delipidated with diethyl ether and apos concentrated with trichloroacetic acid (4.9 mmol/L) and desoxycholic acid (3.6 mmol/L).[22] Apo AI and B were isolated from other apos by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 4-5-10% discontinuous gradient.[23] Apos were identified by comparing migration distances with known molecular weight standards (cross-linked phosphorylase b markers, Sigma, St Louis, MO, and electrophoresis calibration kit, Pharmacia LKB, Biotechnology, Piscataway, NJ). Apo bands were excised from polyacrylamide gels and dried in vacuum for 1 to 2 hours (RC 10-10 Jouan, Saint Herblain, France). The desiccated gel slices were hydrolyzed with 1 mL of 4 mol/L HCl (Sigma, St. Quentin Fallavier, France) at 110°C for 24 hours. As previously discussed,[11] hydrolysis is a critical step because all other proteins are also hydrolyzed and then can dilute the tracer. To avoid any contamination with outside sources that could alter the ratio of labeled versus unlabeled, procedures should be handled carefully, and only new vessels were used. It must be also pointed out that gentle hydrolysis must be performed with deuterated tracers because of the possibility of hydrogen exchange when too harsh conditions are applied.[24] Hydrolysates were then evaporated to dryness, and the amino acids were purified by cation exchange chromatography using a Temex 50W-X8 resin (Bio-Rad, Richmond, CA). Amino acids and plasma leucine were esterified with propanol/acetyl chloride and further derivatized using heptafluorobutyric anhydride (Fluka Chemie AG, Buchs, Switzerland) prior to analysis. Samples were analyzed for isotopic enrichment of leucine using gas chromatography coupled-mass spectrometry (GC-MS) as previously described.[18,19] Briefly, chromatographic separations were performed on a 30 m $\times$ 0.25 mm internal diameter (id) DB-5 capillary column (J&W Scientific, Rancho Cordova, CA). The column temperature was as follows: initial temperature was held at 80°C, then increased at 10°C/min to a final temperature of 180°C. Electron-impact gas chromatography-mass spectrometry was performed on a 5890 gas chromatograph connected with a 5971A quadrupole mass spectrometer (Hewlett-Packard, Palo Alto, CA). The isotopic ratio was determined by selected ion-monitoring at m/z 282 and 285. Results were expressed as tracer/tracee ratio for modeling analysis.[25]

Unesterified cholesterol and cholesteryl ester were isolated from lipoproteins as previously described.[26] Briefly, lipid extraction was performed with chloroform-methanol (2:1, vol/vol) according to Folch et al.[27] Unesterified cholesterol and cholesteryl ester were separated by chromatography on silicic acid[28] using microcolumns (Sep Pack cartridges; Waters Corporation, Milford, MA) The cholesterol samples were derivatized with a mixture of acetic anhydride (500 μL)-pyridine (150 μL) (Aldrich, Saint Quentin, France). The samples were heated to 90°C for 10 minutes. After cooling to room temperature, the derivatizing reagents were evaporated under nitrogen, and the residue was dissolved in hexane. The low ^{13}C-enrichments in cholesterol were below the limit of sensitivity of GCMS and were therefore measured using gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS) with a Finnigan Mat Delta S isotope ratio mass spectrometer coupled to a HP 5890 series II gas chromatograph (Hewlett-Packard) with a DB-1 capillary column (30 m; 0.32 mm id; 0.25 μm film thickness; J&W Scientific). The temperature of the injection port was 280°C, and the oven temperature was initially set at 70°C, then increased at 50°C/min up to 280°C and was held for 15 minutes at 280°C. The samples were injected in splitless mode.

The cholesterol peak eluted from the GC column was introduced on line into a combustion furnace, which operated at 940°C. The furnace is a nonporous alumina tube packed with a wire of copper, nickel, and platinum. Combustion water was removed by passing the effluent through a water-permeable Nafion tube. CO_2 was then driven in the electron impact ion source. In the source CO_2+ ions were formed and signals from major ions m/z 44 ([$^{12}CO_2$]), 45 ([$^{13}CO_2$] + [$^{12}C^{16}O^{17}O$]), and 46 ([$^{12}C^{16}O^{18}O$] + [$^{12}C^{17}O^{17}O$] + [$^{13}C^{16}O^{17}O$]) were monitored simultaneously and continuously by 3 Faraday cup detectors. The same samples were analyzed in triplicate by GC-C-IRMS, and measurements were highly reproducible ($<$0.5%).

Isotopic enrichment was expressed relatively to pulse peaks of CO_2 reference, calibrated against the international standard (Pee Dee Belemnite [PDB]).

Isotopic calculations. The isotopic abundance in ^{13}C in cholesterol is given by the GC-C-IRMS software expressed as δ 0/00

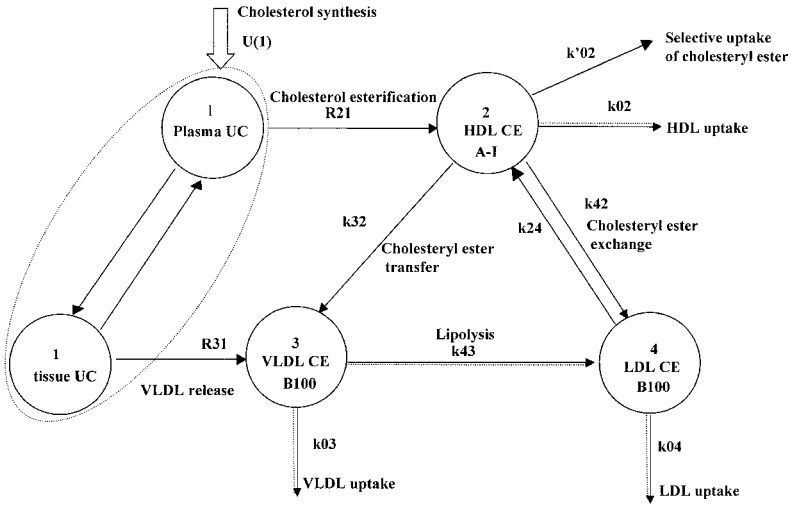

Fig 1. Model of plasma cholesteryl ester (CE) turnover. Using this model, parameters were simultaneously identified from apo B-100, apo AI, and cholesterol data. Dotted arrows represent processes involved in turnover of both apos and cholesterol. For these processes, parameters were common for protein (HDL apo A-I, VLDL, and LDL apo B-100) and cholesterol models. Exchange of unesterified cholesterol (UC) in compartment 1 between lipoproteins and between lipoproteins and tissues, too rapid for constant rate determination (see text) appears in the model without rate constant.

versus the international standard PDB as: $\delta^{13}C$ (0/00) = (R*sample*-R*std*)/R*std*)*1,000, where R is the $^{13}C/^{12}C$ ratio in the sample or the international standard PDB (R*std* = 0.0112372).

Therefore, R*sample* = R*std**[1+(0.001*δ^{13}C)]. The observed fractional abundance F (atom%) was calculated as: F = 100Rstd *[(0.001δ^{13}Csample)+1]. The isotopic enrichment E (atom percent excess) was calculated as: E = Fsample-Fbasal value, where Fsample j and Fbasal values are the observed fractional abundance in the sample at time j and at baseline, respectively. E = 100Rstd*(0.001δ^{13}Csample+1) and APE = atom% sample - atom% basal value (Atom Percent Excess).

Cholesterol concentrations in samples were measured using commercially available enzymatic kits (Boehringer, Mannheim, Germany). Apo B-100 in VLDL and LDL and plasma apo A-I concentrations were measured in samples by immunonephelometry (Behring, Rueil Malmaison, France) as previously described.[29]

Modeling Methods

The apo and cholesterol data were subjected to compartmental analysis using the Simulation, Analysis and Modeling (SAAMII) program.[30] The model used (Fig 1) takes into account the metabolism of both apos (A-I and B-100) and cholesterol. It was derived from a previous model developed for radioisotopes[9] and was adapted to stable isotope labeling. This model described 2 pools for apo B-100 (VLDL-B-100 and LDL-B-100). Each pool described a class of apo B-100–containing lipoprotein. The pools were linked by precursor-product relationships. This model was submitted to 1 input in VLDL pool and has been previously validated in normolipidemic men during tracer perfusion.[18] To describe the metabolism of HDL apo A-I, a single pool model was used.[29] Three compartments represent the metabolism of plasma cholesteryl ester: LDL, HDL, and VLDL. The internal relationships of the system consisted of exchange of cholesteryl ester between LDL and HDL (k24, k42) and unidirectional movement from HDL to VLDL (k32), as previously validated.[8,9] Lipolysis of VLDL established a unidirectional movement of cholesteryl ester from VLDL fraction towards LDL (k43). Cholesteryl ester input into the system consisted of influx by LCAT reaction in HDL (R21) and by VLDL release (R31). Output from the system was achieved by efflux from LDL (k04) and VLDL (k03), which are dependent on apo B-100 metabolism of these lipoproteins. Efflux of cholesteryl ester from HDL occurred dependently of the clearance of apo A-I though HDL catabolism (k02) or independently of this process by selective uptake of cholesteryl ester (k'02). Modeling analysis was performed as described[11] using forcing function to take into account time course of labeling input in cholesteryl ester from unesterified cholesterol and in apo B-100 and apo A-I from plasma leucine.[8,11,19] Parameters were simultaneously identified from apo B-100, apo A-I, and cholesterol data.

As commonly accepted regarding lipoprotein modeling,[31] model validity was checked by statistical analysis using run test[32] for assessing the goodness of fit and using F test[33] and Akaike information criterion[34] for comparison of models.

Consecutively to identification of the model, fluxes of processes in microgram/hour of unesterified cholesterol equivalent per milliliter of plasma were calculated by multiplying the rate constant of the process (in h^{-1}) by the involved mass (in μg/mL of plasma) and used for comparison between each pathway by which cholesteryl ester is leaving the plasma pool.

RESULTS

The concentrations of cholesterol in lipoproteins, apo AI in plasma, and apo B-100 in VLDL and LDL were measured before and 3 sampling times during isotope infusion. Because no significant variations were observed between measurements, all subjects were considered in steady state throughout the study. The plasma apo AI concentration was 1.32 ± 0.11 g/L, and the apo B-100 was, respectively, 54 ± 21 in VLDL and 467 ± 105 mg/L in LDL.

Cholesterol masses for lipoprotein fractions are shown in Table 1. For the 3 subjects, cholesteryl ester represented about 2 thirds of total plasma cholesterol, and 70% of cholesteryl ester was transported by LDL, 25% by HDL, and 5% by VLDL.

The time course of D3-leucine enrichments are presented in Fig 2 for plasma leucine, apos A-I and B-100, and time course of ^{13}C-enrichments in unesterified cholesterol and cholesteryl ester is shown in Fig 3. Regarding leucine enrichment, the data are similar with those obtained in the laboratory in normolipidemic subjects for apos B-100[18] and A-I.[29] The labeled leucine enrichment increased rapidly in VLDL apo B-100 and more slowly in LDL apo B-100 and HDL apo AI, reflecting the lower turnover constant rates of these lipoproteins. The time course of ^{13}C enrichment in plasma unesterified cholesterol was virtually identical in VLDL, LDL, and HDL during the experiment (data

Table 1. Cholesterol Masses in Lipoprotein Fractions and Turnover Parameters of Plasma Cholesteryl Ester Metabolism

	Subject 1	Subject 2	Subject 3
Cholesterol mass[a]			
Plasma total cholesterol	2,060	1,815	1,715
Plasma unesterified cholesterol	720	630	550
VLDL cholesteryl ester	90	85	65
LDL cholesteryl ester	960	840	790
HDL cholesteryl ester	290	260	310
Absolute net influx of cholesteryl ester in plasma[b]			
R21	31.7 ± 1.5*	37.6 ± 5.4	27.2 ± 1.2
R31	0.7 ± 0.4	0.2 ± 0.1	0.3 ± 0.15
Rate constant[c]			
K02	0.006 ± 0.002	0.007 ± 0.001	0.012 ± 0.003
K′02	0.010 ± 0.003	0.020 ± 0.010	0.009 ± 0.005
K32	0.11 ± 0.08	0.10 ± 0.005	0.07 ± 0.04
K24	0.12 ± 0.02	0.08 ± 0.01	0.19 ± 0.02
K42	0.41 ± 0.07	0.26 ± 0.03	0.48 ± 0.05
K03	0.04 ± 0.02	0.01 ± 0.005	0.06 ± 0.02
K43	0.30 ± 0.09	0.37 ± 0.02	0.27 ± 0.01
K04	0.027 ± 0.008	0.033 ± 0.001	0.024 ± 0.001

NOTE. Results were expressed in (a) micrograms of unesterified cholesterol equivalent per milliliter of plasma, (b) micrograms of unesterified cholesterol equivalent per milliliter of plasma per hour, and (c) pool per hour.

*Represents the identified values ± standard deviation as obtained by iterative least-squares fitting.

not shown). Therefore, only enrichments of plasma unesterified cholesterol are presented in Fig 3 and were used as forcing function for the labeling of cholesteryl ester in the model analysis. The order of labeling appearance in plasma cholesteryl ester is shown in Fig 3. Labeling appeared first in unesterified cholesterol, then in the cholesteryl ester:HDL, followed by VLDL, and later by LDL. The apo and cholesterol curves were analyzed using the multicompartmental model of Fig 1.

The parameters related to cholesterol metabolism are shown in Table 1. HDL cholesteryl ester was transferred towards VLDL at the rate of 0.11, 0.10, and 0.07 h^{-1}. HDL cholesteryl ester was transferred towards LDL at the rate of 0.41, 0.26, and 0.48 h^{-1}.

The fractional catabolic rate (FCR) of VLDL (k03+k43) and LDL (k04) apo B-100 was, respectively, 0.34 and 0.027 h^{-1} for subject 1, 0.37 h^{-1} and 0.033 h^{-1} for subject 2, and 0.33 and 0.024 h^{-1} for subject 3. The FCR of HDL apo A-I was 0.006, 0.007, and 0.012 h^{-1} (Table 1).

Fluxes of cholesteryl ester input into the plasma are given in Table 1. The major part of the cholesteryl ester entered plasma through esterification in HDL (more than 95%). Cholesteryl ester left plasma mainly as apo B-100–containing lipoproteins (respectively, 89%, 81%, and 82%). The fate of HDL cholesteryl ester was predominantly the exchange with LDL (76%, 67%, and 84%). In term of net fluxes, the fate of HDL cholesterol ester was predominantly the transfer towards VLDL (87%, 79%, and 77%) rather than direct removal from HDL (13%, 21%, and 23%, with approximately equal parts for apo AI-dependent and independent catabolism, k02 and k'02, mean value 0.008 and 0.013 h^{-1}, respectively). VLDL cholesteryl ester was mainly converted into LDL cholesteryl ester (88%, 97%, and 82%).

DISCUSSION

To our knowledge, the current study is first to use a dual stable isotope approach to assess lipoprotein kinetics through endogenous labeling of both their cholesterol moiety and their protein moiety. Constant infusion of both ^{13}C-acetate and deuterated leucine in our experimental conditions enabled us to obtain measurable deuterium enrichments by GC-MS for plasma leucine, apos A-I and B-100, as well as measurable ^{13}C enrichment by GC-C-IRMS for unesterified cholesterol and cholesteryl ester in the VLDL, LDL, and HDL.

The model used for stable isotopes in this study was identical compared with the model developed with radiotracers,[8,9] but

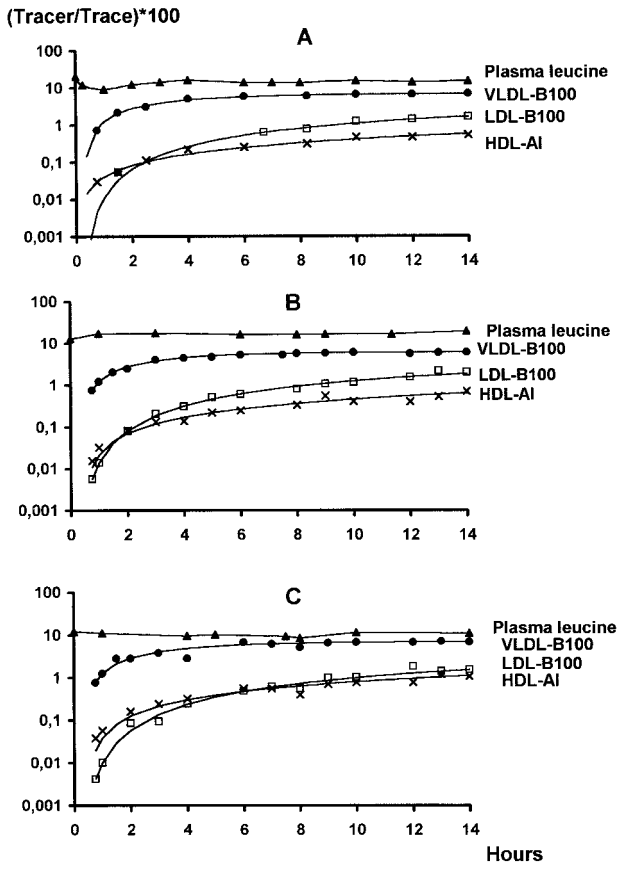

Fig 2. Time course (in semilogarithmic scale) of tracer/tracee ratio in plasma leucine, apo B-100 of VLDL and LDL, and HDL apo A-I during an infusion of deuterated leucine for subject 1 (A), subject 2 (B), and subject 3 (C). Points represent data values. Lines represent curves fitted using the model of Fig 1 for apos and forcing function used for modeling for plasma leucine.

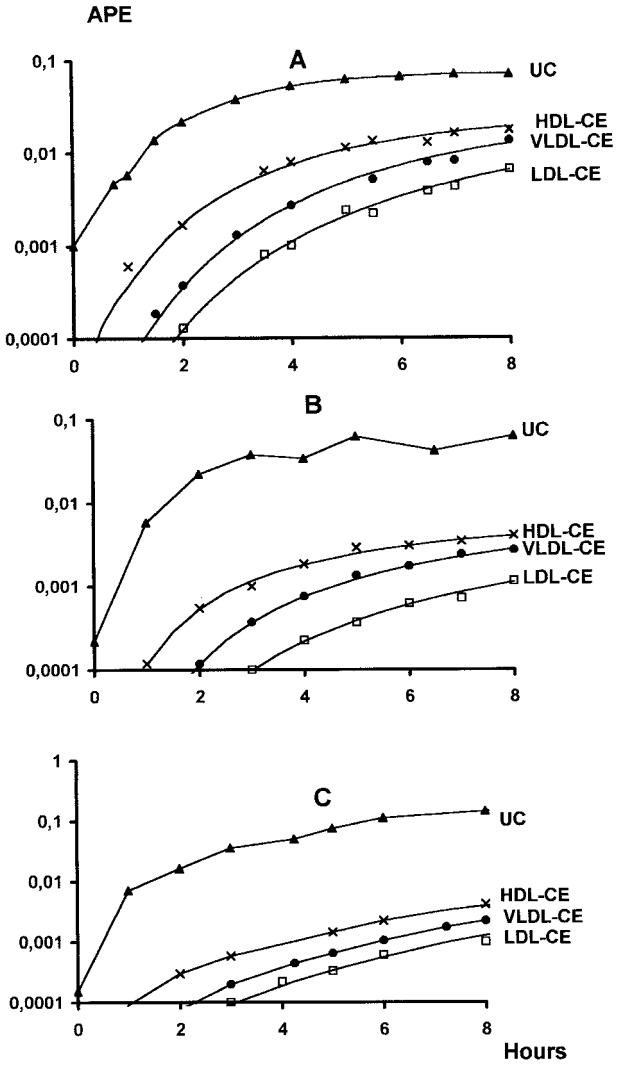

Fig 3. Time course (in semilogarithmic scale) of ^{13}C enrichment (in atom percent excess [APE]) in plasma UC and CE of VLDL, LDL, and HDL during an infusion of ^{13}C-acetate for subject 1 (A), subject 2 (B), and subject 3 (C). Points represent data values. Lines represent curves fitted using the model of Fig 1 for CE and forcing function used for modeling for unesterified cholesterol.

was adapted to endogenous labeling conditions. The order of labeling appearance in lipoproteins agrees with the proposed model structure. In this model, labeling of plasma cholesterol appeared by the way of unesterified cholesterol synthesis, followed by esterification in the HDL and direct cholesteryl ester release into VLDL. Transfers of cholesteryl ester from HDL to VLDL also contributed to the labeling of these lipoproteins. The enrichment of LDL occurred by VLDL lipolysis and transfer from HDL towards LDL. The VLDL release (R31) was introduced in the model because the VLDL cholesteryl ester data could not be entirely fitted from an HDL cholesteryl ester precursor. Thus, fitting required input from another source of cholesteryl ester with a high level of labeling during the first experimental hours. This was achieved by inserting a tissue unesterified cholesterol source in rapid equilibrium with lipoprotein unesterified cholesterol and represented as part of compartment 1 (Fig 1). This is in agreement with the rapid equilibrium previously observed between unesterified cholesterol in liver and plasma in animals during various labeling approaches.[35] Thus, plasma unesterified cholesterol was used as forcing function to represent labeling input from unesterified cholesterol into HDL and VLDL cholesteryl ester.

Coefficient of variations for most of the processes are lower than 30% of the parameter values (Table 1), suggesting the used model is consistent.[31] However, for some of the parameters (net output of HDL cholesteryl ester k'02 and k32, release and direct removal of VLDL R31 and k03), the values showed higher standard deviations (30% to 70% of the identified value). Better coefficients could be obtained by cholesterol kinetics during a longer time, possibly after interruption of the perfusion.[36] Such a tracer methodologic approach could raise the problem of a system study in no steady state conditions.

Although intermediate-density lipoprotein (IDL) were included in LDL fractions in our study, the data obtained for apo B-100 were in the same range as those routinely obtained in normolipidemic subjects using simple apo labeling with stable isotopes after separation of IDL from LDL in our laboratory[18] or by other investigators in recent studies.[37,38] Despite FCR being markedly different for IDL and LDL,[18] the very low IDL concentration (20-fold lower than LDL[18]) involves no consequence on rate constants of apo B-100 turnover parameters. However, IDL isolation could be needed in pathologic conditions resulting in high amounts of IDL.

The data obtained for apo AI were in the same range as those routinely obtained in normolipidemic subjects using simple apo labeling with stable isotopes in our laboratory[29] or by other investigators in recent studies.[39,40] Regarding cholesterol, among the 3 classes of lipoproteins studied, the HDL contained the most rapidly renewed cholesteryl ester (mean of 0.53 h^{-1} *v* 0.35 and 0.16, respectively, for VLDL and LDL). Yet, 75% of the HDL cholesteryl ester turnover corresponds to a seemingly futile cycle of bidirectional exchange with LDL. This is in agreement with a previous study on HDL cholesteryl ester turnover using radiotracers in normolipidemic subjects.[41] For the 3 subjects, the rate transfer of HDL cholesterol towards VLDL was in the same range (0.11, 0.10, and 0.007 h^{-1}) as obtained with radioisotope labeling (0.11 $\pm$ 0.03 h^{-1},[9] 0.12 $\pm$ 0.01 h^{-1}).[8] The transfer rate of HDL cholesteryl ester towards LDL measured (0.41, 0.26, and 0.48 h^{-1}) was also in the same range as compared with radioisotope studies (0.43 $\pm$ 0.18 h^{-1},[9] 0.24 $\pm$ 0.12 h^{-1}).[8] The transfer rate of LDL cholesteryl ester towards HDL measured (0.12, 0.08, and 0.19 h^{-1}) was also in the same range as compared with in vitro studies (0.08 to 0.12 h^{-1}).[42] As in previous studies,[8,9] this transfer occurs under strict cholesterol exchange conditions (zero mass balance). Comparison of cholesteryl ester transfer obtained in our study can be made with in vitro data from methods involving measurements of cholesteryl ester masses transferred from HDL to the apo B-100–containing lipoproteins. The latter methods do not take into account bidirectional exchange of cholesteryl ester between HDL and LDL, but only net mass transfer. The values calculated from data obtained by this method in normolipidemic subjects (near 5% h^{-1})[43] are lower, but in the same range as our data (7%, 10%, and 11% h^{-1}).

The obtained curves in the present study yielded the same quantitative information as those obtained by others using protein and cholesterol radiolabeling with the same model.[8,9] Moreover, compared with the previous studies, our protocol allowed for assessment of the influx rate of esterification in the plasma through LCAT reaction (R21, Table 1) as well. The flux of LCAT-derived cholesteryl ester was estimated by our approach as 32 μg of unesterified cholesterol equivalent per milliliter of plasma per hour, ie, 85 $\mu mol \cdot h^{-1}$ per liter of plasma. This value was in the range of in vitro data (50 to 120 $\mu mol \cdot h^{-1} \cdot L^{-1}$)[44] The rate constant of the reaction can be calculated from influx rate taking into account the involved mass of plasma unesterified cholesterol. The obtained values, 4.5%, 6%, and 5% h^{-1}, respectively, for the 3 subjects are close to those obtained in normolipidemic men by in vitro methods (5% to 6% h^{-1}).[45] As previously reported with other labeling conditions,[8,9,17] the values obtained by our method support the view that in fasting normolipidemic men the essential part of VLDL cholesteryl ester is derived by transfer from HDL (over 95%) rather than from direct hepatic synthesis. As previously suggested by other investigators,[17] the efflux of plasma cholesteryl ester is essentially derived from apo B-100–containing particles after CETP-mediated transfer (80% to 90%) rather than from direct HDL and HDL cholesteryl ester removal.

In conclusion, the current approach proved to be suitable to quantify in vivo the different processes involved in RCT in humans, ie, transfer of cholesteryl ester from HDL towards apo B-100–containing lipoproteins, conversion of VLDL into LDL, the part of the VLDL, LDL, and HDL in the final steps of RCT. Protocols previously published using radiotracer allowed obtaining these parameters. The use of endogenous labeling of cholesterol, however, also determines in vivo the rate of esterification through LCAT reaction, and the use of stable isotopes improves the feasibility of the studies in ethical and safety conditions.

REFERENCES

1. Fielding C, Fielding PE: Molecular physiology of reverse cholesterol transport. J Lipid Res 36:211-228, 1995
2. Williams DL, Conelly MA, Temel RE, et al: Scavenger receptor BI and cholesterol trafficking. Curr Opin Lipidol 10:329-339, 1999
3. Morton RE: Cholesteryl ester transfer protein and its plasma regulator: Lipid transfer inhibitor protein. Curr Opin Lipidol 10:321-327, 1999
4. Gordon T, Castelli WP, Hjortland MC, et al: High density lipoprotein as a protective factor against coronary heart disease. Am J Med 62:707-714, 1977
5. Bruce C, Chouinard RA, Tall AR: Plasma lipid transfer proteins, HDL and reverse cholesterol transport. Annu Rev Nutr 18:297-330, 1998
6. Stein O, Stein Y: Atheroprotective mechanisms of HDL. Atherosclerosis 144:285-301, 1999
7. Lagrost L: Determination of the mass concentration and the activity of the plasma cholesteryl ester transfer protein (CETP). Methods Mol Biol 110:231-241, 1998
8. Monroe P, Vlahcevic ZR, Swell L: In vivo evaluation of lipoprotein cholesterol ester metabolism in patients with liver disease. Gastroenterology 85:820-829, 1983
9. Magot T, Malmendier C, Ouguerram K, et al: In vivo effect of simvastatin on lipoprotein cholesteryl ester metabolism in normocholesterolemic volunteers. Clin Chim Acta 196:59-68, 1991
10. Malmendier C, Lontie JF, Delcroix C, et al: The effect of simvastatin on receptor-dependent low density lipoprotein catabolism in normocholesterolemic volunteers. Atherosclerosis 80:101-109, 1989
11. Maugeais C, Ouguerram K, Krempf M, et al: Kinetic study of apoB100 containing lipoprotein metabolism using amino acids labeled with stable isotopes: Methodological aspects. Clin Chem Lab Med 36:739-745, 1998
12. Marsh JB, Welty FK, Schaefer EJ: Stable isotope turnover of apolipoproteins of high density lipoproteins in humans. Curr Opin Lipidol 11:261-266, 2000
13. Ostlund RE, Matthews DE: [^{13}C] cholesterol as a tacer for studies of cholesterol metabolism in humans. J Lipid Res 34:1825-1831, 1993
14. Jones PJH, Ausman LM, Croll DH, et al: Validation of deuterium incorporation against sterol balance for measurement of human cholesterol biosynthesis. J Lipid Res 39:1111-1117, 1998
15. Ferezou J, Rautureau J, Coste T, et al: Cholesterol turnover in human plasma lipoproteins: Studies with stable and radioactive isotopes. Am J Clin Nutr 36:235-244, 1982
16. Goldberg DI, Beltz WF, Pittman RC: Evaluation of pathways for the cellular uptake of high density lipoprotein cholesterol esters in rabbits. J Clin Invest 87:331-346, 1991
17. Malloy LK, VandenBroek JM, Zech LE, et al: HDL esterified cholesterol uptake by tissues is negligible in vivo in Man. Arteriosclerosis 10:776a, 1990 (abst)
18. Maugeais C, Ouguerram K, Krempf M, et al: A minimal model to study apolipoprotein B-containing lipoprotein metabolism in human using stable isotopes. Diabetes Metab 22:1-7, 1996
19. Frenais R, Ouguerram K, Maugeais C, et al: High density lipoprotein apolipoprotein A-I kinetics in NIDDM: A stable isotope study. Diabetologia 40:578-583, 1997
20. Poupon RE, Ouguerram K, Chrétien Y, et al: Cholesterol-lowering effect of ursodeoxycholic acid in patients with primary biliary cirrhosis. Hepatology 17:577-582, 1993
21. Tersptra AHM, Woodward CJH, Sanchez-Muniz FJ: Improved techniques for separation of serum lipoproteins by density gradient ultracentrifugation: Visualization by prestaining and rapid separation of serum lipoproteins from small volumes of serum. Anal Biochem 11: 149-157, 1981
22. Mindham PA, Mayes PA: A simple and rapid method for the preparation of apolipoprotein for electrophoresis. J Lipid Res 33:1084-1088, 1992
23. Ouguerram K, Magot T, Lutton: Metabolism of intestinal triglyceride-rich lipoproteins in the genetically hypercholesterolemic rat (Rico). Atherosclerosis 931:201-208, 1992
24. Maugeais C, Ouguerram K, Maugeais P, et al: Comparison of [5,5,5-2H3] leucine and [ring-2H5] phenylalanine tracers for the measurement of human apolipoprotein B100 kinetics. J Mass Spectrom 30:478-484, 1995
25. Cobelli C, Toffolo G, Foster DM: Tracer-to-trace ratio for analysis of stable isotope tracer data: Link with radioactive kinetic formalism. Am J Physiol 262:E968-E975, 1992
26. Ouguerram K, Magot T, Lutton C: Metabolism of plasma lipoproteins in the genetically hypercholesterolemic rat (Rico). Metabolism 45:4-11, 1996
27. Folch L, Lees M, Stahley GH: A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem 226:497-509, 1957

28. Hirsh J, Ahrens ED: The separation of complex lipid mixtures by use of silicic acid chromatography. J Biol Chem 233:311-320, 1958

29. Frenais R, Ouguerram K, Maugeais C, et al: Apolipoprotein A-I kinetics in heterozygous familial hypercholesterolemia: A stable isotope study. J Lipid Res 40:1506-1511, 1999

30. Barrett PHR, Bell BM, Cobelli C, et al: SAAMII: Simulation, analysis and modeling software for tracer and pharmacokinetic studies. Metabolism 47:484-492, 1998

31. Pont F, Duvillard L, Vergès B, et al: Development of compartmental models in stable-isotope experiments. Application to lipid metabolism. Arterioscler Thromb Vasc Biol 18:853-860, 1998

32. Sokal RR, Rohlf FJ: Miscellaneous methods, in Freeman WH (ed): Biometry, the Principles and Practice of Statistics in Biological Research, ed 3. New York, NY, Freeman, 1995, 797-803

33. Boxenbaum HG, Tiegelman N, Elastoff RM: Statistical estimations in pharmacokinetics. J Pharm Biopharm 2:123-148, 1974

34. Akaike H: A new look at the statistical model identification. IEE Trans Automat Control AC 19:710-716, 1974

35. Magot T, Verneau C, Lutton C, et al: Origin and fate of cholesterol in rat plasma lipoproteins in vivo: I. Qualitative analysis. Ann Nutr Metab 29:147-159, 1985

36. Parhoffer KG, Barrett PHR, Bier DM, et al: Determination of kinetic parameters of apolipoprotein B metabolism using amino acids labeled with stable isotopes. J Lipid Res 32:1311-1323, 1991

37. Duvillard L, Pont F, Florentin E, et al: Metabolic abnormalities of apolipoprotein B-containing lipoproteins in non-insulin-dependent diabetes: A stable isotope kinetic study. Eur J Clin Invest 30:685-694, 2000

38. Welty FK, Lichtenstein AH, Barrett PHB, et al: Effects of apoE genotype on apoB-48 and apoB-100 kinetics with stable isotopes in humans. Arterioscler Thromb Vasc Biol 20:1807-1810, 2000

39. El Khalil L, Majd Z, Bakir R, et al: Fish eye disease: Structural and in vivo metabolic abnormalities of HDL. Metabolism 46:474-483, 1997

40. Schaefer JR, Winkler K, Schweer H, et al: Increased production of HDL apoA-I in homozygous familial defective apoB100. Arterioscler Thromb Vasc Biol 20:1796-1799, 2000

41. Schwartz CC, Berman M, Vlahcevic ZR, et al: Multicompartmental analysis of metabolism in man. Qualitative kinetic evaluation of precursor sources and turnover of HDL cholesterol esters. J Clin Invest 70:867-876, 1982

42. Barter PJ, Jones MJ: Kinetic studies of the transfer of esterified cholesterol between human plasma low and high density lipoproteins. Atherosclerosis 34:67-74, 1979

43. Bagdade JG, Ritter MC, Subbaiah PV: Accelerated cholesteryl ester transfer in plasma of patients with hypercholesterolemia. J Clin Invest 87:1259-1265, 1991

44. Glomset JA: The plasma lecithin: cholesterol acyltransferase. J Lipid Res 9:155-167, 1968

45. Yen FT, Nishida T: Rapid labelling of lipoproteins in plasma with radioactive cholesterol. Application for measurement of plasma cholesterol esterification. J Lipid Res 31:349-353, 1990