ApoA-II kinetics in humans using endogenous labeling with stable isotopes: slower turnover of apoA-II compared with the exogenous radiotracer method

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Abstract ApoA-II is a major apolipoprotein constituent of high density lipoproteins (HDL) and may play an important role in lipoprotein metabolism and predisposition to atherosclerosis. Previous radiotracer kinetic studies have suggested that the metabolism of apoA-II in humans may be different than the metabolism of apoA-I, the major HDL apolipoprotein. In the present study, we have used an endogenous labeling technique using stable isotopically labeled amino acids to study apoA-II metabolism and compared the results to those obtained by a simultaneous exogenous radiotracer labeling method. Seven subjects with HDL cholesterol levels ranging from 9 to 93 mg/dl and apoA-II levels from 13 to 60 mg/dl were investigated in this study. [$^{13}C_6$]phenylalanine and ^{131}I-labeled apoA-II were simultaneously administered as a primed-constant infusion and a bolus injection, respectively. In the endogenous labeling study, plateau tracer/tracee ratios of VLDL apoB-100 were used as estimates for the precursor pool tracer/tracee ratios for apoA-II synthesis. Residence times of apoA-II using these two independent methods were found to be highly correlated (r = 0.973, P < 0.0002). These results indicate that the endogenous labeling of apoA-II using stable isotopically labeled amino acids is a reasonable alternative to the conventional exogenous radiotracer labeling method for the investigation of apoA-II turnover. However, under the conditions of our experimental design and modeling strategy, the apoA-II residence times as determined by endogenous labeling were significantly longer (mean 5.33 days) than by exogenous radiotracer (mean 4.65 days). This suggests that apoA-II turnover may be even slower than believed based on radiotracer studies, and further supports the concept that HDL containing apoA-II are metabolized differently than HDL without apoA-II.—**Ikewaki, K., L. A. Zech, H. B. Brewer, Jr., and D. J. Rader.** ApoA-II kinetics in humans using endogenous labeling with stable isotopes: slower turnover of apoA-II compared with the exogenous radiotracer method. *J. Lipid Res.* 1996. **37:** 399–407.

Supplementary key words high density lipoproteins · atherosclerosis

Plasma concentrations of high density lipoprotein (HDL) cholesterol have been shown to be inversely associated with the incidence of coronary artery disease (CAD) (1, 2). However, the mechanism by which HDL may exert a direct protective effect against the development of atherosclerosis is not well understood. The two major proteins in HDL are apoA-I and apoA-II. ApoA-I is required for normal HDL biosynthesis and promotes esterification of HDL free cholesterol by lecithin:cholesterol acyltransferase (LCAT) (3). Many epidemiologic studies have demonstrated that plasma apoA-I concentrations correlate inversely with the incidence of CAD (4–6). In contrast, the correlation of apoA-II levels and CAD is inconsistent and the role of apoA-II in HDL metabolism remains unclear (1, 4). Some evidence suggests that apoA-II may even be pro-atherogenic. A mouse strain whose apoA-II production was higher relative to other strains had higher levels of HDL cholesterol, but also more atherosclerosis (7). Transgenic mice overexpressing mouse apoA-II develop atherosclerosis on a normal chow diet (8). Transgenic mice overexpressing human apoA-I alone are protected from diet-induced atherosclerosis, whereas those overexpressing both human apoA-I and human apoA-II develop atherosclerosis despite having similar HDL cholesterol levels (9).

Metabolic studies in humans using radiotracer methods have suggested that apoA-I and apoA-II have differ-

Abbreviations: IEF, isoelectric focusing; GC–MS, gas chromatography–mass spectrometry; HDL, high density lipoprotein; FCR, fractional catabolic rate; FSR, fractional synthetic rate; RT, residence time; PR, production rate; PBS, phosphate-buffered saline.

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ent metabolic pathways and regulation. Most studies have demonstrated that plasma concentrations of apoA-I are mainly regulated by the rate of catabolism (10–12). In contrast, levels of apoA-II are determined solely by the rate of production (11, 12). There exist two major HDL subclasses: particles containing apoA-I but not apoA-II (LpA-I), and those that contain both apoA-I and apoA-II (LpA-I:A-II) (13). We have shown previously that apoA-I on LpA-I is catabolized faster than apoA-I on LpA-I:A-II (14). Furthermore, in apoA-I and apoA-II kinetic studies in 50 normolipidemic subjects, we found that LpA-I levels were inversely correlated with the rate of apoA-I catabolism, whereas LpA-I:A-II levels were correlated with the rate of apoA-II production (15). These results indicate that apoA-I and apoA-II have distinct metabolic pathways and that apoA-II production rates may be a determinant of atherosclerosis risk.

Apolipoprotein kinetic studies have been performed mainly using exogenous radioiodination and this methodology has contributed significantly to our current understanding of HDL metabolism. More recently, endogenous labeling techniques using stable isotopes have been applied to apolipoprotein kinetic studies (16, 17). We previously reported that apoA-I kinetic parameters determined by endogenous labeling compared favorably with those determined by the radiotracer method (18). However, apoA-II kinetics have not been assessed using endogenous labeling. In this study, we assessed apoA-II kinetics using both methods simultaneously in subjects whose apoA-II levels varied over a wide range. ApoA-II residence times as assessed by the two methods were well correlated, indicating that endogenous labeling with stable isotopes can be considered as a reasonable alternative to assess differences in apoA-II kinetics in humans. However, the apoA-II residence times determined by the stable isotope method were significantly longer than those determined by the radiotracer method. In conclusion: *1)* apoA-II turnover can be quantitated in humans using endogenous labeling with stable isotopes and correlates with radioisotope studies; and *2)* apoA-II turnover may be slower than previously believed based on radiotracer studies, suggesting that the in vivo metabolic differences between apoA-I and apoA-II may be substantial.

METHODS

Study subjects

Four normolipidemic, one hyperalphalipoproteinemic, and two hypoalphalipoproteinemic subjects were recruited for this study. ApoA-I kinetic studies in the four normolipidemic subjects were previously reported (18). The hyperalphalipidemic subject was not deficient either in cholesteryl ester transfer protein activity or hepatic lipase activity. The two hypolipidemic subjects, previously reported elsewhere (19), were not deficient in LCAT activity. The study subjects were admitted to the Clinical Center of the National Institutes of Health. All study subjects had normal fasting glucose levels and normal thyroid, liver, and renal function, were on no medications affecting lipid metabolism, and they gave informed consent to the study protocol approved by the Clinical Research Subpanel of the National Heart, Lung, and Blood Institute.

Isolation and iodination of apoA-II

ApoA-II was isolated from normal HDL by gel permeation chromatography and ion exchange chromatography as previously described (20) and stored at -20°C. Lyophilized apoA-II was redissolved in a buffer of 6 M guanidine-HCl and 1 M glycine (pH 8.5), and iodinated with ^{131}I by a modification of the iodine monochloride method (14). Approximately 0.5 mol iodine was incor-

TABLE 1. Characteristics of study subjects

Subjects	Sex	Age	BMI	TC	TG	HDL-C	ApoA-I	ApoA-II
		yr			mg/dl			
1	M	63	26.7	284	68	93	251	60
2	F	22	22.0	185	96	53	138	37
3	F	20	19.3	163	40	66	140	31
4	F	21	21.9	144	62	49	131	28
5	F	21	19.3	176	61	53	128	27
6	F	51	25.6	297	247	10	37	21
7	M	38	25.7	143	148	9	33	13
Mean		34	22.9	199	103	48	123	31
SEM		7	1.2	24	27	11	28	6

Abbreviations: BMI, body mass index; TC, total cholesterol; TG, triglyceride; HDL-C, HDL-cholesterol; apoA-I, apolipoprotein A-I; apoA-II, apolipoprotein A-II.

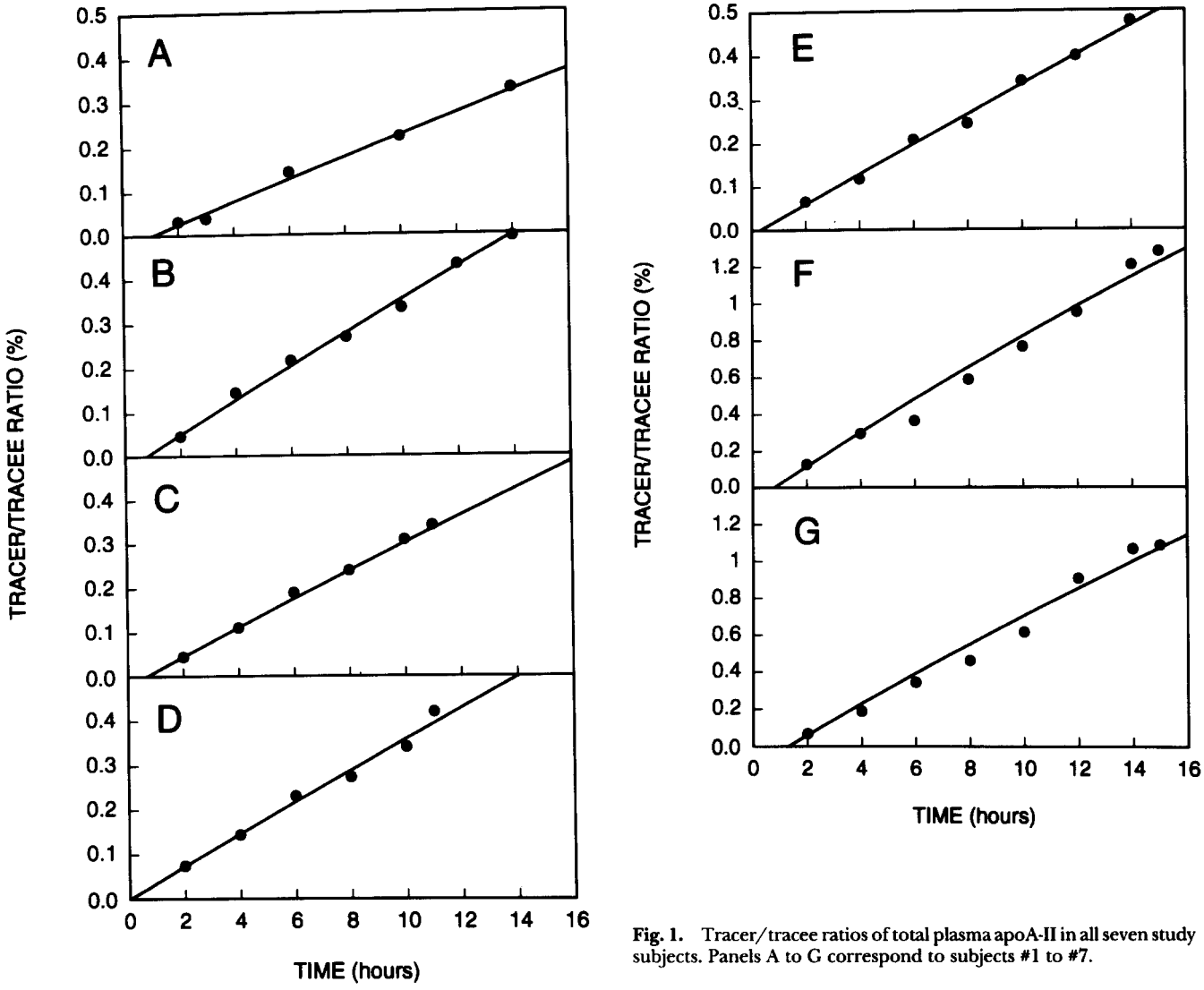

Fig. 1. Tracer/tracee ratios of total plasma apoA-II in all seven study subjects. Panels A to G correspond to subjects #1 to #7.

porated per mol of protein. Iodinated apoA-II was reassociated with autologous plasma and immediately dialyzed at 4°C for 12 h against four changes of PBS containing 0.01% EDTA. Human serum albumin was added to a final concentration of 5% (wt/vol). Samples were sterile-filtered through a 0.22 μm Millipore filter and tested for pyrogens and sterility prior to injection.

Study protocol

Three days prior to the study, subjects were placed on an isoweight diet containing 47% carbohydrate, 37% fat, 16% protein, 200 mg of cholesterol per 1000 Kcal, and a polyunsaturated to saturated fat ratio of 0.3. Meals were given three times per day and the diet was continued during the metabolic study. One day prior to the study, the subjects were started on potassium iodide (900 mg) in divided doses and this was continued throughout the study period. After a 12-h fast, the subjects were injected with up to 25 μCi of ^{131}I-labeled apoA-II. [$^{13}C_6$]phenylalanine (ring-$^{13}C_6$ 99%, Cambridge Isotope Laboratories, Woburn, MA) was simultaneously administered as a priming bolus of 600 μg/kg, immediately followed by a constant infusion of 12 μg/kg per min over a period of 12 to 14 h. During the infusion, meals were served in equal small portions every 2 h. Blood samples were obtained 10 min after the injection and then at 1, 2, 3, 4, 5, 6, 8, 10, 12, 14, 18, 24, and 36 h, daily through day 5, and day 7, 9, 11, 14. Urine was collected continuously throughout the study.

Blood samples (20 ml) were drawn into the tubes containing EDTA at a final concentration of 0.1%. The blood was kept on ice and the plasma was immediately separated by centrifugation at 2300 rpm for 30 min at 4°C. Sodium azide and aprotinin were added to the plasma at final concentrations of 0.05% and 200 KIU/ml, respectively. Radioactivity in plasma, urine,

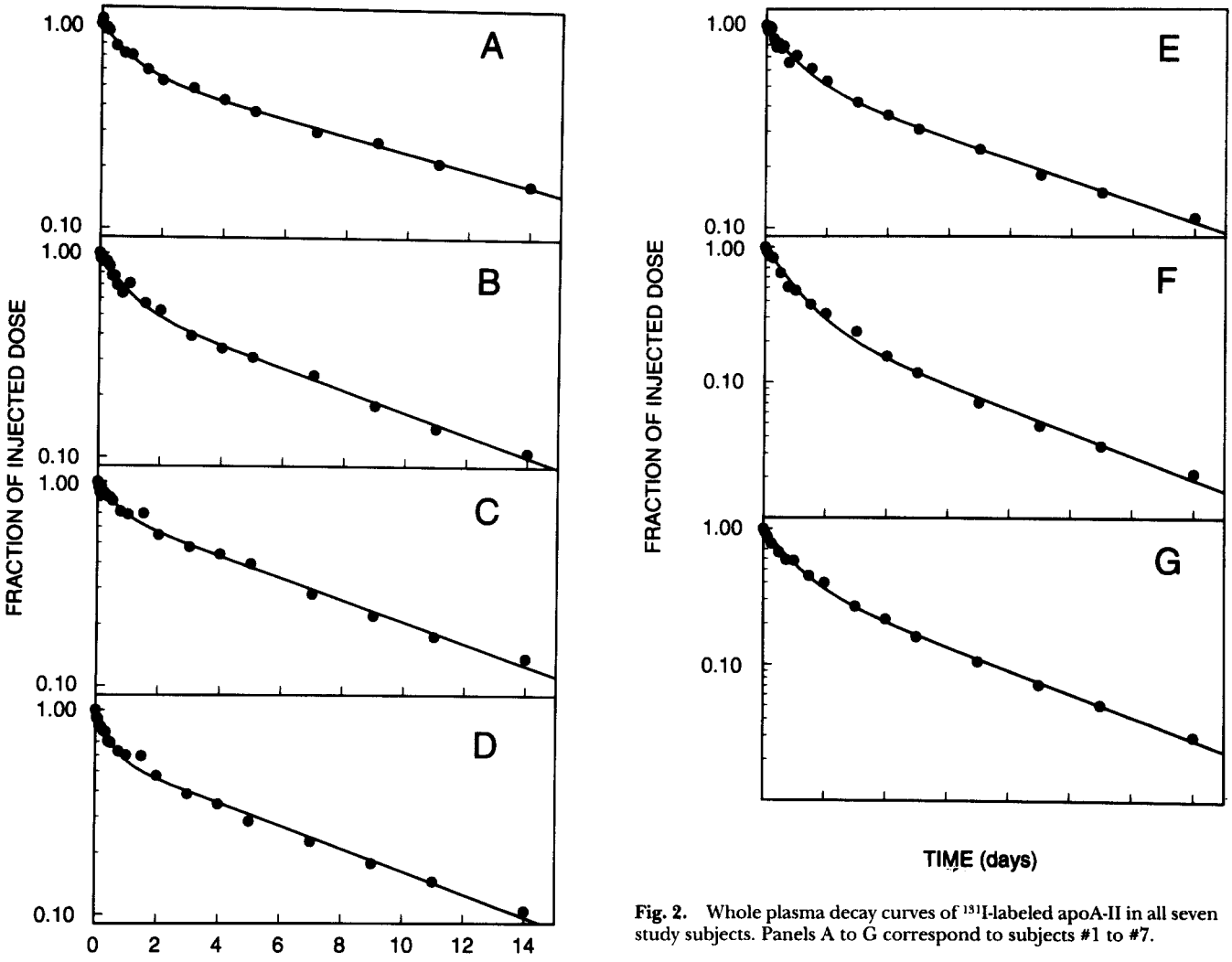

Fig. 2. Whole plasma decay curves of ^{131}I-labeled apoA-II in all seven study subjects. Panels A to G correspond to subjects #1 to #7.

and isolated lipoprotein fractions was quantitated in a Packard Cobra gamma counter (Packard Instrument Co., Downers Grove, IL).

Isolation of lipoproteins and apolipoproteins

Total plasma lipoproteins were isolated on a TL-100 ultracentrifuge (Beckman Instruments, Palo Alto, CA) using a TLA-100.3 rotor at 100,000 rpm for 10 h after 1 ml of plasma was adjusted at the density of 1.25 g/ml with solid KBr. VLDL was isolated from 5 ml of plasma by ultracentrifugation using 40.3 rotor at 39,000 rpm for 20 h as previously described (21). Lipoproteins were dialyzed against 10 mM ammonium bicarbonate, lyophilized, and delipidated. VLDL apoB-100 was isolated by preparative gradient NaDoSO$_4$-PAGE (5–15%) (22) and apoA-II was isolated by preparative isoelectric focusing (pH 4–6) (23).

Determination of isotopic enrichment

Samples were prepared for gas chromatograph–mass spectrometric (GC–MS) analysis as reported previously (18, 22, 24). Briefly, apolipoprotein bands were cut from gels and dried overnight (90°C), then hydrolyzed in 6 N HCl (Ultrapure grade, J. T. Baker, Inc., Phillipsburg, NJ) at 110°C for 24 h. The protein hydrolysates were lyophilized in a Speed-Vac evaporator (Savant Instrument, Inc., Farmingdale, NY). Free amino acids were purified from plasma or protein hydrolysates by cation exchange chromatography (AG-50W-X8, Bio-Rad Laboratories, Richmond, CA), then derivatized to the N-heptafluorobutyryl isobutyl esters, and analyzed by GC–MS on a Finnigan MAT 4500 (Finnigan MAT, San Jose, CA) in the chemical ionization mode, using isobutane as the reagent gas. Selective ion monitoring at 418 *m/z* for unlabeled phenylalanine and 424 *m/z* for [$^{13}C_6$]phenylalanine was used to determine the isotope ratio. Each sample was analyzed at least 3 times. Enrichment was

calculated from the isotope ratio using the method of Cobelli, Toffolo, and Foster (25). Enrichment was then converted to tracer/tracee ratios by using the formula: tracer/tracee ratio = $e(t)/(e_I - e(t))$, where $e(t)$ is the enrichment of each sample at time t, and e_I is the enrichment of the infusate, which was 99% in this study.

Analysis of kinetic data

Stable isotope studies. A monoexponential function was fitted to the tracer/tracee ratio curves of VLDL apoB-100 and total apoA-II using SAAM31, as previously reported (18, 26). The function was defined as $A(t) = Ap(1-e^{(-k(t-d))})$, where A(t) is the tracer/tracee ratio at time t, Ap is the tracer/tracee ratio of precursor pool for the apolipoprotein of interest, d is the delay time, and k is the fractional synthetic rate (FSR). The VLDL apoB-100 plateau tracer/tracee ratio was used as the precursor pool enrichment for apoA-II, based on data demonstrating that apoA-II is synthesized primarily or exclusively in the liver (27, 28).

Radiotracer studies. Plasma radioactivity decay curves were constructed as the fraction of injected dose using the 10 min plasma counts as the initial time, and were fitted to two-exponential functions using the SAAM31 program (26). The residence time (RT) was obtained from the area under the plasma decay curve. The fractional catabolic rate (FCR) is the reciprocal of the RT and is equal to the FSR at a steady state.

Analytical methods

Plasma total cholesterol and triglyceride levels were determined by automated enzymatic techniques on an Abbott VPSS analyzer (Abbott Labs, North Chicago, IL). HDL cholesterol was measured by dextran sulfate precipitation (29). Plasma apoA-II concentrations were quantitated using an immunoturbidometric assay (Boehringer-Mannheim, Mannheim, Germany).

TABLE 2. Residence times of apoA-II determined by endogenous stable isotope and exogenous radiotracer labeling methods

Subjects	Residence Times		Difference
	Stable Isotope	Radiotracer	
	days		
1	7.45	6.80	0.65
2	5.10	4.95	0.15
3	6.85	5.92	0.93
4	5.99	4.79	1.20
5	6.40	5.10	1.30
6	2.59	2.27	0.32
7	2.98	2.75	0.23
Mean	5.33	4.65	0.68[a]
SEM	0.72	0.61	0.18

[a]Significantly different ($P = 0.009$) between two labeling methods by paired *t*-test.

RESULTS

Characterization of the study subjects is shown in **Table 1.** HDL cholesterol levels ranged from 9 to 93 mg/dl with the mean concentration of 48 mg/dl, plasma apoA-II from 13 to 60 mg/dl with the mean of 31 mg/dl. These values were stable throughout the study period, indicating that the studies were performed under steady state conditions.

The tracer/tracee ratios of plasma free plasma phenylalanine in some of the study subjects (#2–5) were previously reported (18) and were relatively constant throughout the infusion period. The VLDL apoB-100 tracer/tracee curves increased rapidly and reached plateau within 8 h. The tracer/tracee curves of apoA-II in all seven subjects are shown in **Fig. 1.** The tracer/tracee curves were nearly linear during the infusion period and were fitted to a monoexponential function. The fractional standard deviations of the apoA-II FSRs as determined by the monoexponential function analysis ranged from 0.02 to 0.33 with a mean of 0.13.

The plasma decay curves of ^{131}I-labeled apoA-II in all study subjects are shown in **Fig. 2.** The plasma decay curves were fitted to a two-exponential function.

The residence times (RT) of apoA-II using stable isotope and radiotracer methods are summarized in **Table 2.** The mean (± SEM) apoA-II RT determined using the VLDL apoB-100 plateau tracer/tracee ratio as an estimate of the apoA-II precursor pool enrichment was 5.33 ± 0.72 days. The mean residence time of apoA-II was 4.65 ± 0.61 days (Table 2). In all individuals, the residence time obtained by the endogenous labeling with stable isotope was longer than that obtained by the exogenous radiotracer method. Therefore, the mean apoA-II residence time by stable isotope determination was significantly longer (0.68 days) than that using radiotracer methodology ($P = 0.009$ by paired *t*-test).

Finally, the correlation of the residence times between stable isotope and radiotracer methods is shown in **Fig. 3.** Residence times using the stable isotope method were found to be well correlated with those by radiotracer method ($r = 0.973$, $P < 0.0002$).

DISCUSSION

ApoA-II is a major protein constituent of HDL, but its role in lipoprotein metabolism has not been defined. Unlike apoA-I, apoA-II does not appear to have a direct role in preventing atherosclerosis. For example, al-

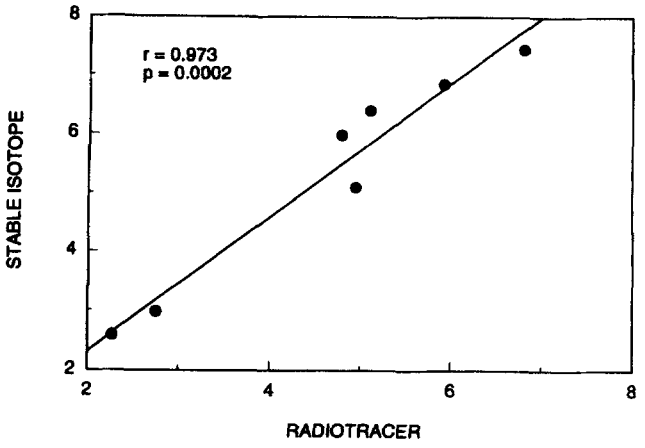

Fig. 3. Correlation of apoA-II residence times between radiotracer and stable isotope methods in the seven study subjects.

though individuals with apoA-I deficiency are at increased risk of developing premature CAD (30, 31), apoA-II deficiency does not appear to predispose to increased risk (32). Furthermore, transgenic mice overexpressing human apoA-I are resistant to diet-induced atherosclerosis (33), whereas the same mice also expressing human apoA-II are not protected despite similar HDL cholesterol levels (9). In fact, some animal studies have suggested that apoA-II may even increase atherosclerosis. A mouse strain with higher levels of HDL cholesterol and apoA-II has increased aortic fatty streak development (7). Furthermore, transgenic mice overexpressing mouse apoA-II have been reported to develop atherosclerosis on a chow diet (8). Given the potential role of apoA-II in lipoprotein metabolism and atherosclerosis, it is important to understand the metabolic pathways of apoA-II in humans.

Exogenous labeling methodology using radiotracer has been utilized for the study of apoA-II kinetics in humans (34, 35), but there is currently much interest in the use of endogenous labeling with stable isotopes for the study of apolipoprotein kinetics in humans (24). We previously reported that the endogenous labeling of apoA-I with stable isotopes resulted in apoA-I kinetic parameters that were highly comparable to those obtained by a simultaneously performed exogenous radiotracer study (18). The major goal of the present study was to investigate apoA-II kinetics in humans using endogenous labeling and compare the results with those obtained by simultaneous radiotracer studies. Although we previously found apoA-I kinetics to be comparable using the two methods, the fact that apoA-I is derived from both intestine and liver complicated the analysis of the endogenous labeling study. In contrast to apoA-I, apoA-II is made only in the liver (28), thus in theory allowing more precise determination of the intracellular precursor pool enrichment. Therefore, we were interested in directly and simultaneously comparing the turnover of apoA-II as assessed by endogenous versus exogenous labeling.

In this study, we used an analytical method similar to that used for apoA-I (18) to determine apoA-II kinetic parameters. First, the plateau tracer/tracee ratio of VLDL apoB-100, a liver-derived protein, was assumed to be identical to the intracellular precursor pool tracer/tracee ratio for apoA-II synthesis, based on the observations that apoA-II is synthesized primarily in the liver (28). Second, a monoexponential function was used to calculate apoA-II kinetic parameters. The duration of our study did not permit the use of a two-exponential function, as the apoA-II tracer/tracee ratio increased virtually in a linear fashion over the time period of the constant infusion.

We found that apoA-II kinetic parameters were highly correlated between these two independent methods. This is consistent with our previous report in subjects with LCAT deficiency (36) demonstrating accelerated apoA-II catabolism by both endogenous labeling and radiotracer methods. Therefore, these methods appear to be comparable in their ability to determine relative rates of apoA-II turnover among different individuals. However, in this study we found a consistent and significant difference in the absolute values of apoA-II turnover in the form of slower rates of catabolism as determined by the endogenous labeling method.

The reason for the relatively slower rates of apoA-II catabolism as determined by endogenous labeling is not entirely clear. Purification and radioiodination of apoA-II may not be completely free from any modifications of the protein (35). Osborne et al. (37) reported a radioiodinated species of apoA-I that was different from native apoA-I in molecular properties, indicating possible modifications of the apolipoprotein during purification and/or labeling processes. As apoA-II has more affinity to lipids than apoA-I, there exists a possibility that apoA-II may be denatured by delipidation during purification, resulting in tracer being catabolized at a faster rate. Unlike apoA-I which is present as a monomer, apoA-II forms both monomer, and homo- and heterodimers in human plasma (38). We have recently reported that the rate of catabolism is directly associated with the form in which apoA-II was present (39). Therefore, exogenously labeled apoA-II may have a different distribution among these forms, resulting in a faster overall catabolism compared with native (endogenously labeled) apoA-II. Another explanation may reside in the report by Beltz et al. (40), who showed differences in apoB-100 kinetics between endogenous and exogenous labeling methods. Because VLDL consists of various subpopulations that are different in metabolic behavior,

they concluded that VLDL populations labeled by the endogenous technique may be different from those labeled exogenously. One potential explanation for our results is that exogenously labeled apoA-II may reassociate with lipoproteins in a distribution different from that of endogenously labeled apoA-II.

Our results suggest that apoA-II turnover in humans may be substantially slower than previously believed. **Table 3** shows residence times of apoA-I and apoA-II as determined by both exogenous radiotracer and endogenous stable isotope labeling methods in four subjects (subject #2 to #5 in the present study) in which apoA-I and apoA-II kinetics were simultaneously investigated. The mean apoA-II residence time was found to be 8% longer ($P = 0.06$) by the radiotracer method and 18% longer ($P = 0.004$) by the stable isotope method. These observations are consistent with previous radiotracer studies showing that apoA-II turnover is similar to but slightly slower than that of apoA-I (10, 11, 34, 35), and further confirm this concept by two independent labeling methods. Kinetic studies in humans have confirmed that apoA-I associated with apoA-II is catabolized more slowly than apoA-I not associated with apoA-II (14). The finding that apoA-II turnover may, in fact, be considerably slower than that of apoA-I provides further evidence that HDL has several metabolic pathways that may be influenced, at least in part, by the presence of apoA-II on the HDL particle.

Although slower rate of turnover of apoA-II is of potential importance, caution must be exercised in the interpretation of the kinetic data due to the different study designs and analytic methods using the exogenous and endogenous labeling approaches. First, the duration of the endogenous labeling study (16 h) may not be long enough to accurately estimate kinetic parameters of apoA-II, a slowly turning-over protein. Second, the mono-exponential model used for the endogenous stable isotope study may not be optimal for apoA-II tracer/tracee data, based on the fact that apoA-II kinetics are complex, as evident from the plasma disappearance curve of the exogenous labeled tracer. However, it should also be noted that compartmental models reasonably representing apoA-II kinetics have not been established yet (41, 42). Third, a potential limitation resides in our assumption that the precursor pool enrichment for apoA-II synthesis is equal to the plateau enrichment of VLDL apoB-100. Accumulating evidence has shown that apoB-100 secretion is determined not only by the amount synthesized but by the partitioning of newly synthesized apoB-100 between degradation in the endoplasmic reticulum and secretion. Therefore, our assumption that the precursor pool enrichment for apoA-II synthesis is equal to the plateau enrichment of VLDL apoB-100 may not be accurate. Fourth, the validity of the exogenous labeling method depends on an assumption that protein structure and property is not altered by either isolation, labeling, or subsequent reassociation. In summary, both the endogenous and exogenous methods involve assumptions requiring that the absolute kinetic parameters be interpreted with caution. Nevertheless, the excellent correlation between these two entirely different methods lends credence to the concept that the relative apoA-II turnover rates can be reasonably estimated by either technique.

In summary, the present study has established that endogenous labeling of apoA-II using stable isotopically labeled amino acid results in apoA-II residence times that are significantly longer, but highly correlated with those derived from the exogenous radiotracer labeling method. This indicates that endogenous stable isotope labeling is a practical alternative to the conventional exogenous radiotracer method for the investigation of

TABLE 3. Comparison of residence times of apoA-I and apoA-II determined by endogenous stable isotope and exogenous radiotracer labeling methods

	Residence Times			
	Radiotracer		Stable Isotope	
Subjects	ApoA-I[a]	ApoA-II	ApoA-I[a]	ApoA-II
	days			
2	4.42	4.95	4.61	5.10
3	5.08	5.92	5.75	6.85
4	4.59	4.79	4.98	5.99
5	5.10	5.10	5.23	6.40
Mean	4.80	5.19	5.14	6.09
SEM	0.15	0.22	0.21	0.32
P-value[b]	0.06		0.004	

[a]Data from the previous study (ref. 18).
[b]P-value represents a difference of residence times between apoA-I and apoA-II by paired *t*-test.

apoA-II kinetics in humans. The suggestion that apoA-II turnover may, in fact, be considerably longer than previously believed based on radiotracer studies must be confirmed, but could have significant implications for the understanding of the different pathways of HDL metabolism.

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