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A comparison of two methods to investigate the metabolism of human apolipoproteins A-I and and A-II

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Summary Two methods are compared for measuring the kinetic parameters of apolipoprotein A-I and A-II metabolism in human plasma. In the first, high density lipoprotein apoproteins were radioiodinated in situ in the lipoprotein particle (endogenous apoprotein labeling) while in the second, individually labeled apolipoprotein A-I or A-II was incorporated into the particle by in vitro incubation (exogenous apoprotein labeling). The catabolic clearance rate of exogenously labeled apolipoprotein A-I was consistently faster than that of endogenous apolipoprotein A-I. Conversely, endogenously and exogenously labeled apolipoprotein A-II were catabolized at identical rates. The fractional plasma clearance rates of endogenous apolipoproteins A-I and A-II were the same.

Supplementary key words high density lipoprotein metabolism

Human high density lipoproteins (HDL) contain two major and several minor apoprotein constituents (1, 2). The former, apolipoproteins A-I and A-II, exhibit cooperativity in maintaining the structural integrity of the lipoprotein (3). ApoA-I is also an activator of lecithin: cholesterol acyltransferase (4, 5) and may regulate the lipid content of membranes (6, 7) and, thus, their fluidity (8). Despite the evident importance of these proteins, little is known of their metabolism in humans. Blum and co-workers (9) have examined their catabolism by labeling HDL with ^{125}I and following the rate of plasma clearance of each protein isolated from the labeled lipoprotein at intervals after its intravascular injection. They demonstrated that in normal subjects the specific activity decay curves of the two proteins were superimposable, even when the ratio of apoA-I to apoA-II in the plasma was perturbed by dietary or pharmacologic means.

In this report we describe and evaluate an alternative and less laborious method of examining apoA-I and apoA-II metabolism in humans. The procedure involves: *a*) incorporation of radiolabeled apoA-I or apoA-II into HDL by in vitro incubation; *b*) isolation of the labeled lipoprotein by ultracentrifugation at d 1.21 g/ml; *c*) intravascular injection of the tracer; and *d*) sequential measurement of the rate of removal of radioactivity from the plasma at daily intervals for 10–14 days following the injection.

Methods

Materials

Pooled blood was collected from healthy human volunteers in 1/100 volume of 1.0 M Tris-HCl buffer, pH 7.0, containing 1.0% disodium EDTA. The plasma was separated by low speed centrifugation and fractionated immediately.

$Na^{125}I$ and $Na^{131}I$ were purchased from Amersham-Searle, Arlington Heights, IL; Sephadex and Blue Dextran 2000 from Pharmacia Fine Chemicals, Piscataway, NJ; 6% agarose gels (Bio-Gel A5m, 200–400

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mesh) from Bio-Rad Laboratories, Richmond, CA; and 1,1,3,3-tetramethylurea from Sigma Chemical Company, St. Louis, MO. All other chemicals were reagent grade.

HDL preparation

Human plasma HDL was prepared by column chromatography as described elsewhere (10, 11), dialyzed against 0.05 M sodium barbital buffer, pH 8.6, and its molarity was determined by protein analysis (12), assuming that each mole of HDL contains 125,000 g of protein. The preparation was free of LDL and albumin contamination as determined by electroimmunoassay (13).

Purification of apolipoproteins A-I and A-II

ApoA-I and apoA-II were prepared by standard chromatographic procedures (14) from the HDL after lyophilization and delipidation with ether–ethanol (15). The isolated apoproteins were then dialyzed against 0.1 M NH_4HCO_3 (pH 8.6), lyophilized, and stored at −70°C. Their purity was confirmed by amino acid analysis, acrylamide gel electrophoresis, and crossed immunoelectrophoresis (11). Preparations found to be impure by any of these criteria were rechromatographed. Each apoA-II preparation was chromatographed at least twice before use. Specific antibodies were raised in rabbits against the purified apoproteins (16).

Labeling of HDL, apoA-I and apoA-II

HDL was labeled with ^{125}I or ^{131}I by the McFarlane technique (17) as modified by Bilheimer, Eisenberg, and Levy (18). No more than 4% of the radioactivity was found in the lipid fraction and, of the remainder, 50–60% was associated with apoA-I and 30–40% with apoA-II as determined by acrylamide gel electrophoresis (19) and Sephadex G-150 gel filtration (14).

ApoA-I and apoA-II were radioiodinated by a modification of the McFarlane technique (17). The reaction mixture contained 50 nmol of apoprotein and 100 nmol of ^{125}ICl (2.0 mCi) in 1.5 ml of 1.0 M glycine buffer, pH 10.0. Immediately after mixing, the iodinated protein was separated from unbound radioiodide by gel filtration through a 1.0 × 25 cm Sephadex G-15 column. The eluting buffer contained 0.1 M Tris-HCl, pH 8.6, 0.15 M NaCl, and 0.01% disodium EDTA. Labeling efficiency was approximately 50%, consequently the resulting I/protein ratio was 1/1. The labeled apoprotein was dialyzed against 0.05 M sodium barbital buffer, pH 8.6, until paper electrophoresis showed that less than 1% free radioiodide remained in the protein preparation.

Metabolic studies

Apolipoprotein A-I metabolism. HDL was isolated from the plasma of two healthy adults (one male, one female) by the chromatographic procedure described elsewhere (10, 11). A 2-ml aliquot, containing 100 nmol of HDL protein, was labeled directly with ^{131}I (18). An equal aliquot was incubated at 37°C for 30 min with 10 nmol of ^{125}I-apoA-I in 8.0 ml of 0.05 M sodium barbital buffer, pH 8.6. At this low apoA-I concentration (1.25 nmol/ml), the apoprotein is essentially monomeric (20) and there is a mole for mole exchange of the free apoprotein with apoA-I in the HDL (11). After labeling, both HDL tracers were purified separately by ultracentrifugal flotation in KBr at d 1.21 g/ml (24 hr, 2.5 × $10^5 g$, 10°C, in a Beckman 65 anglehead rotor) and dialyzed against 0.01 M Tris-HCl, pH 7.0, containing 0.15 M NaCl and 0.01% disodium EDTA. Twenty-five microcuries of each HDL preparation were mixed together, sterilized by filtration through a 0.22-μm cellulose membrane (Millipore Corp., Bedford, MA), and injected intravenously into the donor. All preparations were free of pyrogens and bacterial contamination. Plasma clearance of the ^{125}I- and ^{131}I-apoA-I was measured by the procedure described previously (11). Essentially, total plasma lipoproteins isolated at daily intervals by ultracentrifugation at d 1.21 g/ml were incubated for 30 min at 37°C with an equal volume of tetramethylurea and the resulting tetramethylurea-soluble material was applied to a column of G-150 Sephadex and eluted with 5.4 M urea in 0.1 M Tris-HCl, pH 8.6. The fractions corresponding to apoA-I (Fractions 48–52, **Fig. 1***A* were pooled and the specific activities of the ^{125}I- and ^{131}I-apoA-I were measured by radioactive counting and electroimmunoassay (11). This labeled protein migrated as a single band on sodium dodecyl sulfate polyacrylamide gel electrophoresis (19), coincident with a marker of apoA-I.

In an additional study, HDL was isolated from the plasma of a healthy male volunteer as described above and labeled directly with ^{125}I. Tetramethylurea precipitation and G-150 Sephadex chromatography was then used to isolate ^{125}I-apoA-I from the lipoprotein. After dialysis against 0.05 M sodium barbital buffer (pH 8.6) the labeled apoprotein was reincorporated into freshly prepared HDL from the same subject by the in vitro transfer procedure detailed above. Using identical conditions, an equal aliquot of the subject's HDL was labeled by incubation with ^{131}I-apoA-I. The plasma clearance rates of the apoprotein labeled by these two procedures were compared in the donor subject. The two apoprotein preparations comigrated on sodium dodecyl sulfate polyacrylamide gel electrophoresis (19).

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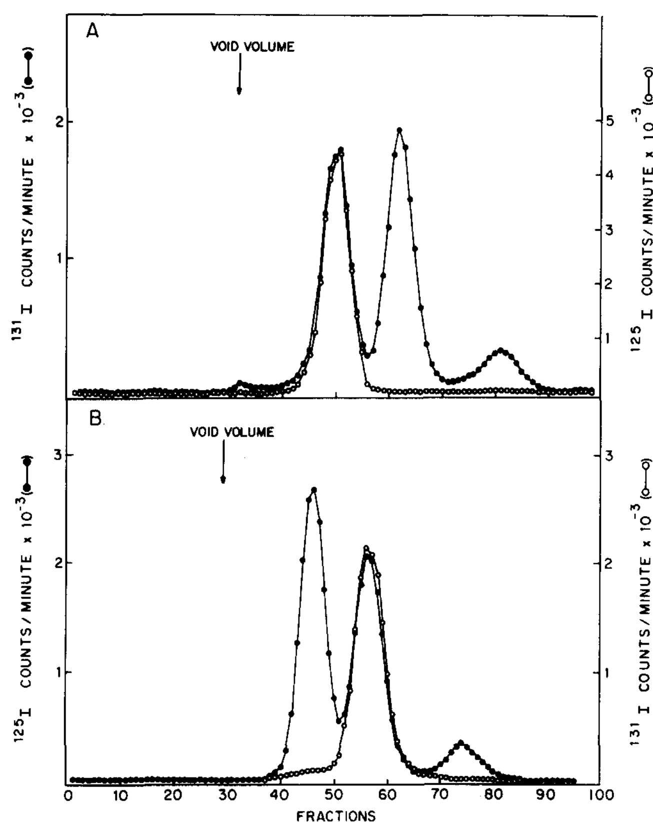

Fig. 1. Separation of radioiodinated apolipoproteins A-I and A-II, prepared from total plasma lipoproteins on columns of Sephadex G-150. The lipoproteins from 6.0 ml of plasma were isolated by ultracentrifugal flotation at d 1.21 g/ml, dialyzed against 0.01 M Tris-HCl, pH 7.0, containing 0.15 M NaCl and 0.01% disodium EDTA, concentrated to 0.25 ml in a Minicon B15 cell (Amicon Corp., Lexington, MA), and the radioactive apoproteins released by addition of an equal volume of tetramethylurea. The tetramethylurea-soluble proteins were fractionated on 1.6 × 100 cm columns of Sephadex G-150 in 5.4 M urea–0.01 M Tris–HCl, pH 8.6, and the radioactivity of the eluate fractions was determined on a Packard Autogamma spectrometer. Panel *A*, Radioactivity profile of total plasma lipoproteins containing ^{131}I-HDL and ^{125}I-apoA-I/HDL; Panel *B*, radioactivity profile of total plasma lipoproteins containing ^{125}I-HDL and ^{131}I-apoA-II/HDL.

Apolipoprotein A-II metabolism. The metabolism of apoA-II labeled in situ in HDL with ^{125}I was compared with that of ^{131}I-apoA-II incorporated into HDL in vitro using conditions of labeling identical to those described for apoA-I. Two subjects were studied (one male, one female). After labeling, mixing, sterilizing, and injecting the radioactive tracers as described above, daily plasma samples were again obtained and treated with tetramethylurea to isolate apoA-I (Fractions 44–47, Fig. 1*B*) and apoA-II (Fractions 55–58, Fig. 1*B*). After dialysis against 0.05 M sodium barbital buffer (pH 8.6), the specific activities of these proteins were determined by radioactive counting and protein measurement using both colorimetric (12) and electroimmunoassay procedures (13) for apoA-I and apoA-II. The apoA-II immunoassay was linear over the range 30–260 μg apoA-II/ml (r = 0.994). The within- and between-batch coefficients of variation for the assay were 4.4% (n = 16) and 3.8% (n = 10), respectively. The specific activities obtained by both methods were essentially the same. As was found before for apoA-I (11), ultracentrifugal isolation and fractionation of the total plasma lipoproteins on columns of 6% agarose (10) at intervals throughout the study showed that more than 95% of the ^{131}I and ^{125}I radioactivity in the plasma of each volunteer was associated with HDL.

Throughout all studies, the participants received 300 mg of KI thrice daily to prevent thyroidal uptake of radioiodide.

Results

The plasma clearances of endogenous (i.e., labeled in situ) and exogenous (i.e., incorporated in vitro) apoA-I in the HDL of the male subject are compared in **Fig. 2*A***. The decay profiles are both consistent with a two-compartment model in which equilibration was achieved within 5 days (the mean correlation coefficients for ^{125}I-apoA-I and ^{131}I-apoA-I specific activity decay after day 5 were 0.995 and 0.992, respectively). The results obtained for the female subject were similar to those for the male. In both cases, it appeared that ^{125}I-apoA-I incorporated into the HDL by in vitro incubation was catabolized faster than the ^{131}I-apoA-I labeled in the intact HDL particle. This was confirmed by mathematical examination of the data using the multicompartmental analytical procedure of Matthews (21). The results (**Table 1**) indicate that, in the male and female subject, respectively, the fractional catabolic rate (i.e., the fraction of the intravascular pool catabolized per day) of the exogenously labeled apoA-I was 50% and 25% faster than that of the endogenously labeled apoprotein. The half-life of endogenously and exogenously labeled apoA-I in the male and female reflected this differential catabolism (the exogenous apoA-I half-life was 81% and 78% of the endogenous value, respectively).

The possibility that these metabolic differences may have arisen from differential uptake of radioiodide into apoA-I free in solution or as a component of HDL was excluded in the study whose results are shown in **Fig. 2*B***. The decay curves of the apoprotein labeled by both of these procedures were identical, as reflected in the calculated fractional clearance rates (Table 1).

The plasma decay curves of endogenously and exogenously labeled apoA-II are shown in **Fig. 3** and are compared to the decay profile of endogenously labeled apoA-I in the same subject. All curves

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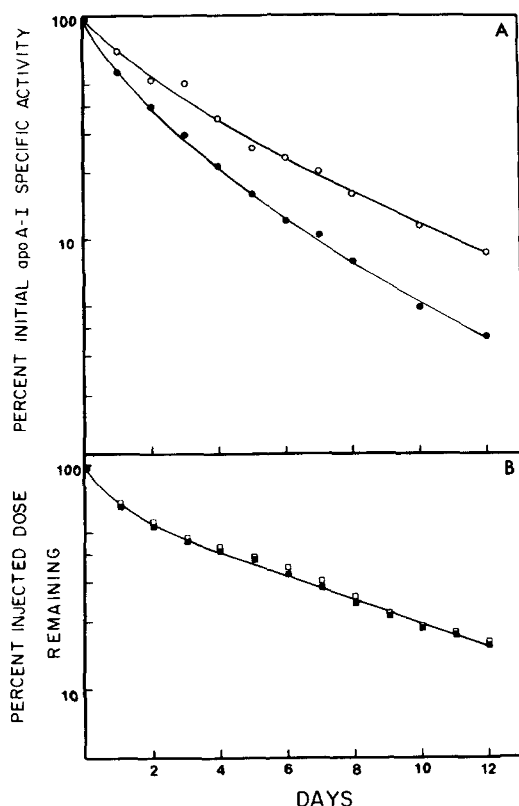

Fig. 2. *A*. Specific activity decay curves of apoA-I in the plasma of subject M.Y. M.Y. was injected intravenously with autologous ^{131}I-HDL and ^{125}I-apoA-I/HDL, and plasma samples were collected at daily intervals for 12 days. ApoA-I was isolated on Sephadex G-150 as described in the legend of Fig. 1, and its ^{131}I and ^{125}I-specific activity was determined by radioactivity counting and electroimmunoassay. ○, ^{131}I-apoA-I specific activity; ●, ^{125}I-apoA-I specific activity. *B*. Plasma decay curves of ^{125}I- and ^{131}I-apoA-I in subject M.D. Autologous HDL was labeled with ^{125}I and delipidated, and the ^{125}I-apoA-I was isolated by column chromatography. The plasma clearance of this protein was compared with that of homologous apoA-I labeled directly with ^{131}I. Both labeled tracers were intercalated separately into the subject's HDL prior to injection. □, Homologous ^{131}I-apoA-I decay curve; ■, autologous ^{125}I-apoA-I decay curve.

are essentially the same, again conforming to a two-compartment model. Mathematical analyses of the curves (Table 1) confirmed this impression. The fractional clearance rates of endogenously and exogenously labeled apoA-II differ by no more than 5% in both subjects and the mean clearance rate of endogenous apoA-I is within 7% of that of apoA-II.

Discussion

The dearth in the literature relative to the metabolism of human apolipoproteins A-I and A-II reflects the difficulties involved in performing such studies. Blum and co-workers (9) have approached the problem by radiolabeling holo-HDL and following its clearance from the plasma, having previously established that the decay curves of apoA-I and apoA-II in

TABLE 1. Fractional catabolic rate of endogenous[a] and exogenous[a] apolipoproteins A-I and A-II in humans

Subject (Sex)	Injected Tracer	Apoprotein Studied	Derived Fractional Catabolic Rate[b] (pools/day)
M.Y. (M)	^{131}I-HDL	^{131}I-apoA-I	0.24
	^{125}I-apoA-I/HDL	^{125}I-apoA-I	0.37
R.H. (M)	^{125}I-HDL	^{125}I-apoA-I	0.34
	^{131}I-apoA-II/HDL	^{125}I-apoA-II	0.31
		^{131}I-apoA-II	0.32
S.B. (F)	^{125}I-HDL	^{125}I-apoA-I	0.23
	^{131}I-apoA-II/HDL	^{125}I-apoA-II	0.22
		^{131}I-apoA-II	0.23
D.E. (F)	^{131}I-HDL	^{131}I-apoA-I	0.24
	^{125}I-apoA-I/HDL	^{125}I-apoA-I	0.30
M.D. (M)	^{125}I-apoA-I/HDL[c]	^{125}I-apoA-I	0.16
	^{131}I-apoA-I/HDL	^{131}I-apoA-I	0.17

[a] Endogenous refers to apoproteins labeled in situ in HDL while exogenous indicates that the apoprotein was bound to HDL by incubation in vitro.
[b] Calculated by the method of Matthews (25).
[c] Endogenous apoA-I labeled in situ in HDL, purified by chromatography, and subsequently intercalated into the lipoprotein by incubation.

the lipoprotein were superimposable. This method has inherent problems. The most important of these is the requirement for daily apoHDL fractionation to obtain accurate specific activity determinations for the individual proteins. Moreover, as the specific activity of the proteins decreases towards the end of the study, the error in its determination is increased at a time when the greatest accuracy is required. Since apoA-I

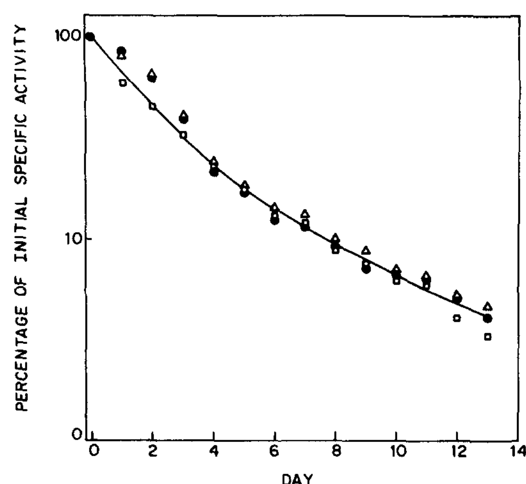

Fig. 3. Specific activity decay curves of apoA-I and apoA-II in the plasma of subject R.H. R.H. was injected intravenously with autologous ^{125}I-HDL and ^{131}I-apoA-II/HDL, and plasma samples were collected at intervals of 13 days. ApoA-I and apoA-II were isolated by gel filtration as described in Fig. 1 legend and their specific activities were determined by radioactivity and protein measurement. △, ^{125}I-apoA-II specific activity; ●, ^{131}I-apoA-II specific activity; □, ^{125}I-apoA-I specific activity.

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and apoA-II decay identically, an approximation of their clearance rates may be obtained by analysis of the total plasma clearance curves. This method, however, ignores the significant contribution made by non-apoA-associated radioactivity to the total apoHDL decay.

In this report we present our experiences with an alternative approach to the problem which was conceived from the long-established finding that the apoproteins of HDL are readily assimilated into their parent lipoprotein in vitro, forming a stable particle (22, 23). By using radioiodinated apoA-I or apoA-II it was possible to prepare specific HDL-labeled tracers to study the metabolism of these apoproteins in humans. Prior to such studies it was important to show that the exogenously labeled apoprotein was metabolically indistinguishable from its endogenous counterpart. In the case of apoA-I, this was not so. Fig. 2*A* indicates that although the clearance of ^{125}I-apoA-I bound to HDL in vitro is very similar to that of the same apoprotein labeled with ^{131}I in situ in the lipoproteins, there is a small but consistent difference in their metabolic handling. Conversely, apoA-II bound in vitro to HDL was metabolically indistinguishable from its counterpart labeled in situ in the particle (Fig. 3, Table 1). Moreover, as noted by Blum et al. (9), the endogenous apoA-II was cleared from the plasma at a rate identical to that of apoA-I in HDL. Consequently, we believe that this apoprotein, bound to HDL in vitro, provides a useful marker for the study of HDL metabolism in man.

The reason for the difference in metabolic clearance of apoA-I labeled in holo-HDL or by the in vitro transfer procedure is not yet apparent but we believe that the available evidence supports the premise that it is a physiological phenomenon. For example, (*a*) ApoA-I under physiological conditions normally exhibits inter-particle transfer (24, 25).
(*b*) We have provided evidence to exclude the possibilities that the discrepancy derives from overiodination of the apoA-I or from its carbamylation (11). Moreover, Fig. 2*B* shows that the apoprotein is catabolized identically, whether labeled free in solution or as an integral part of HDL; nor was there differential handling of autologous and homologous apoA-I in this subject.
(*c*) When incorporated into HDL, the labeled apoprotein remains with the lipoprotein throughout its biological lifetime and exhibits an inter-compartmental distribution that is consistent with its continued association with a high molecular weight species such as HDL (26).
(*d*) ^{125}I-apoA-I incorporated into HDL in vitro is cleared from the plasma at a constant rate, as demonstrated by the urine/plasma radioactivity ratio,[2] indicating that the tracer is metabolically homogeneous.
(*e*) In a study[2] of apoA-I and apoA-II metabolism in ten healthy subjects by the method detailed in this report, the fractional clearance rate of apoA-I was consistently and significantly ($P < 0.01$) faster than that of apoA-II. The mean difference (± 1 SD) was $21 \pm 6\%$. Since we have shown in the present study that the metabolism of exogenous and endogenous apoA-II in HDL are the same, this finding indicates that the metabolism of ^{125}I-apoA-I intercalated into HDL is uniformly faster than that of its counterpart labeled in situ in the lipoprotein.

These data, together with our earlier observation that only two-thirds of the total HDL apoA-I is exchangeable in vitro (11), support the premise that this fraction represents a physiological subpopulation of apoA-I in the lipoprotein particle.

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