Measurement of apolipoprotein A-I in rat high density lipoprotein and in rat plasma by radioimmunoassay

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Abstract A double antibody radioimmunoassay (RIA) for rat apolipoprotein A-I is reported. The ApoA-I isolated from delipidated HDL by gel filtration yielded a single band on polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS), and its amino acid composition resembled that reported by others. ApoA-I was iodinated by lactoperoxidase and the resulting ^{125}I-apoA-I was purified by gel filtration. Up to 93% of ^{125}I-apoA-I was precipitable by antibody and >99% of bound ^{125}I-apoA-I was displaced by "cold" apoA-I. Other rat lipoproteins and apolipoproteins did not react in this system. Human plasmas were also not reactive, nor were dog, goat, and sheep plasmas.

Rat intact HDL displaced ^{125}I-apoA-I in parallel with "cold" apoA-I; however, only about 10% of the apoA-I in intact HDL reacted in the assay. Removal of the lipid from HDL by extraction with ether–ethanol solutions increased the reactivity of apoA-I to levels expected from studies of the apoA-I content of HDL by column chromatography and SDS disc gel electrophoresis. Plasmas from Sprague-Dawley male and female rats also displaced counts in parallel with apoA-I. Apparent plasma levels of apoA-I ranged from 2 to 7 mg/dl in unextracted plasma. When assay incubations were carried out for two hours at 37°C at the beginning of the assay and again right after the addition of the second antibody, similar low results were obtained. Lipid extraction increased apparent levels about 9-fold to 39 ± 8 mg/dl for Sprague-Dawley males (n = 14) and 47 ± 6 mg/dl for females (n = 5). ApoA-I was not lost during extraction. Heating of plasmas to 52°C before assay yielded levels of 26 ± 4 mg/dl. Thus, extraction of plasmas appears to be necessary to obtain accurate levels of apoA-I in this assay system.

Supplementary key words rat high density lipoprotein · apolipoprotein A-I · radioimmunoassay

Because of its manifold functions and complex metabolic fate (1, 2), studies of the structure and metabolism of HDL have great physiologic and pathophysiologic importance. Yet, until recently, convenient assays for the apolipoproteins of HDL were not available (3, 4). We have recently reported a radioimmunoassay for human apoA-I which has proved to be useful in this regard (3). Since the rat is such a frequently used experimental species, we have developed a similar assay for rat apoA-I. This paper details the assay procedure, particularly where it differs from its counterpart for human apoA-I.

METHODS

Isolation of HDL

HDL was isolated by ultracentrifugation from the plasmas of Zucker fatty rats and of lean controls between calculated densities of 1.070 and 1.18 g/ml (5). Ultracentrifugations were carried out for 3×10^8 *g*-min in a 60 Ti rotor and a L2-65B Beckman preparative ultracentrifuge (Spinco Div., Palo Alto, Cal.) (6). Only HDL, but not apoB, albumin, or other serum proteins were detectable in these HDL preparations by immunoelectrophoresis against antirat serum (5, 7) (Behring Diagnostics, Inc., Somerville, N.J.).

Isolation of apoA-I

HDL was dialyzed against 1 mM EDTA pH 8.6 and lyophilized. Delipidation was then carried out with ether–ethanol solutions according to Scanu and Edelstein (8). The resulting ApoHDL was solubilized in 0.1 M Tris, pH 8.6, 8M urea (Tris–urea), and chromatographed on a 2.5 × 90 cm column containing Sephadex G-200, equilibrated with Tris–urea (9). Column chromatographic fractions were monitored at 280 nm. Ten to twenty mg of protein were applied and recovery of protein averaged 95%. Three peaks were routinely obtained. On polyacrylamide disc gel electrophoresis in urea (10) the first peak contained several slowly moving apoproteins; the second, a single major band; and the third, several bands corresponding to apoA-II and apoC. These results resemble those reported by others (11, 12). However, on

Abbreviations: VLDL, LDL, and HDL are very low, low, and high density lipoproteins, respectively. Apolipoproteins of VLDL LDL, and HDL are designated as ApoVLDL, ApoLDL, and ApoHDL. Individual apolipoproteins are designated in analogy to human apolipoproteins (5) as apoA, apoB, etc. TG, triglyceride; chol, cholesterol; RIA, radioimmunoassay; SDS, sodium dodecyl sulfate; ^{125}I-apoA-I, ^{125}I-labeled apoA-I.

TABLE 1. Amino acid composition of rat apoA-I.

	S–D #612	S–D #028	Fatty #055
		moles %	
Lys	10.90	8.15	8.48
His	2.56	2.50	2.71
Arg	5.51	6.34	6.06
Asp	12.35	12.30	12.80
Thre	4.61	4.90	4.87
Ser	4.45	4.17	4.44
Glu	19.11	20.67	20.66
Prol	3.34	2.86	3.01
Gly	6.08	5.22	5.03
Ala	7.41	7.26	6.98
Cys/2	N.D.	N.D.	N.D.
Val	5.30	4.09	4.61
Met	2.54	3.30	3.31
Ile	1.76	1.52	1.52
Leu	10.30	12.27	11.03
Tyr	1.68	1.88	1.99
Phe	2.29	2.51	2.51
Trp	Not done	Not done	Not done

Two preparations isolated from Sprague-Dawley pools and one isolated from a Zucker fatty rat plasma pool are reported. N.D. = none detected.

SDS disc gel electrophoresis (13), peak 2 contained two bands corresponding to apoA-I and the "arginine-rich" apoprotein (14). When peak 2 was pooled as two separate fractions, the ascending portion of peak 2 contained approximately 70% apoA-I and 30% "arginine-rich" apoprotein, whereas the descending portion contained >95% apoA-I (obtained by photometric scanning of the SDS gel and integrating the areas under the individual peaks by planimetry). The descending portions of peak 2 were repurified on the G-200 columns.

ApoA-I prepared in this way migrated as a single band in the appropriate position on disc gel electrophoresis in SDS (13, 14) and yielded a single immunoprecipitin band on immunodiffusion and immunoelectrophoresis against antirat serum. It did not react with antisera raised against the third chromatographic peak containing apoA-II and apoC, against rat apoB, and against other rat serum proteins. Its amino acid composition (**Table 1**) resembled that reported by others (11, 14). (We are grateful to Dr. Ralph Bradshaw of the Department of Biochemistry, Washington University School of Medicine, for the amino acid analyses.) Upon injection of apoA-I into rabbits (see ref. 3 for immunization procedures), antisera were produced which reacted against rat apoA-I, but not against rat LDL (d 1.030–1.050 g/ml), rat apoC isolated from rat VLDL (11), or the third chromatographic peak of HDL on the Sephadex G-200 columns.

To assess the apoA-I content of rat ApoHDL, the three Sephadex G-200 peaks were pooled, dialyzed, and analyzed according to the method of Lowry et al (15) using crystalline bovine serum albumin (BSA) as standards. ApoA-I represented 45% ± 3 (n = 4) of the protein of HDL. The percentage apoA-I was also determined by measuring the areas under the peaks of the elution profile by planimetry. By this method, the percentage apoA-I was 57% ± 3 (n = 4). ApoHDL was also subjected to disc gel electrophoresis in SDS (14). These gels were scanned photometrically and areas under the curves were determined by planimetry (see below). ApoA-I content was 55 and 60% (n = 2). (We realize each of these methods gives only an approximation of the apoA-I content of ApoHDL. Column chromatography is limited by the cross-contamination of the peaks and SDS electrophoresis is limited by the lack of knowledge of the "chromagenicities" of the individual proteins. Nevertheless there is fair agreement between these methods and the RIA as seen below.)

Iodination of apoA-I

0.5–1 mCi of $Na^{125}I$ (carrier free, Amersham Searle, Arlington Heights, Ill.) was dried under N_2 in a small conical test tube. Twenty five μl of 0.05 M phosphate buffer, pH 7.6, was added, followed by 20 μl of apoA-I solution (25 μg protein) and 15 μl of lactoperoxidase (16) (150 μg/ml, Calbiochem, Inc., San Diego, Cal.). The reaction was started with the addition of 2 μl of H_2O_2 (0.44 mM). After 1 min, 500 μl of 0.05 M phosphate buffer was added and the contents of the tube were loaded onto a 0.09 × 30 cm chromatographic column containing Sephadex G-50 (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) equilibrated with barbital buffer (0.05 M, pH 8.6). The first peak corresponding to the void volume contained the iodinated protein and approximately 20% of the counts, and the second broad peak contained the unreacted iodine. The tubes in the first peak were pooled and subjected to gel filtration on a 1.5 × 30 cm column of Sephadex G-75 equilibrated with barbital buffer, 3% BSA (BSA–barbital). This partially pure ^{125}I-apoA-I was stored at 4°C and a second purification through Sephadex G-75 was always carried out within 24 hr of any assay. ^{125}I-apoA-I eluted with chymotrypsinogen, mol wt 25,000, on Sephadex G-75, suggesting that the labeled protein was in monomeric form in BSA–barbital, the assay buffer. The calculated specific radioactivity of fresh ^{125}I-apoA-I preparations was 3–5 μCi/μg protein. Iodinations were carried out every 4–6 weeks.

Lipid extraction or heating of HDL and of plasma for apoA-I assay

In order to assess the apoA-I content of HDL and of plasma accurately, it was necessary to extract their lipids with organic solvents. This was carried out as described previously for the human apoA-I assay (3). 0.1 and 0.2 ml solutions of HDL and 0.1 and 0.2 ml of plasma were extracted with 10 ml of ether–ethanol 2:3 (v/v) overnight. Protein precipitates were washed twice with ether, dried under nitrogen, solubilized in 5–10 ml of 8 M urea and allowed to sit at 15°C for several hours. To avoid any deleterious effects of urea on the assay itself, extracted samples were diluted 1:20 to 1:50 with BSA–barbital before assay. Similar dilutions of 8 M urea added to the B_0 tubes of the assay did not affect precipitability of counts in the assay.

In an attempt to increase the reactivities of rat plasmas in the assay without the need for prior lipid extraction, some plasmas were diluted 1:100 in BSA–barbital and heated to 52°C for 3 hr prior to assay. (We are grateful to Dr. Arthur

Rubenstein of the Department of Medicine, University of Chicago, for this suggestion.)

Other procedures

Triglyceride (17) and cholesterol (18) were determined chemically on extracts prepared according to the method of Carlson (17). Proteins were determined according to Lowry et al (15) using BSA standards. The protein contents of apoA-I standard solutions were also determined by total amino acid analysis, and the masses of apoA-I as determined by the two procedures were compared. Urea disc gels were stained with Coomassie Blue (19) and SDS gels with Amido Black; both were scanned in a Gilford 2400-S recording spectrophotometer at 550 nm (Gilford Instrument Labs., Oberlin, Ohio). Areas under scan peaks were quantified by planimetry.

The assay

Assays were carried out as described for the human assay (3), in test tubes which had been precoated with Siliclad (Clay Adams, Division of Becton-Dickinson Co., Parsippany, N.J.). All dilutions were made in BSA–barbital. A typical tube contained 10–200 μl of sample, 50 μg of non-immune rabbit serum (diluted 1:200), 100 μl of anti apoA-I (diluted 1:500 to 1:2500), 100 μl of ^{125}I-apoA-I (8–12,000 cpm, 1–3 ng), and sufficient BSA–barbital to bring the volume of 500 μl. Blank tubes which contained no anti apoA-I were also included to control for nonspecific precipitation. (This averaged less than 3% of added counts.) Other blank tubes contained only label and buffer, to control for the adsorption of label to glass (this was consistently less than 2% of added counts). In routine assays all reactants were added at the same time. Tubes were incubated for 42 hr at 4°C; second antibody (goat anti rabbit IgG, 50 μl diluted 1:20) was then added and the tubes were incubated for an additional 18 hr. On two occasions tubes were incubated at 37°C for 2 hr and at 4°C for 40 hr (20). After addition of second antibody, tubes were again incubated at 37°C for 2 hr and at 4°C for 14 hr. Results were calculated in a Hewlett-Packard Model 10 tabletop programmable calculator (Hewlett-Packard, Palo Alto, Cal.) using a program provided by the vendor which calculates results of assays from standard curves calculated in terms of the logit function (21). Results were sometimes also calculated in terms of percent precipitability (B/T) or in terms of B/B_0. B = ^{125}I-apoA-I cpm precipitated minus cpm in nonspecific precipitate. B_0 = ^{125}I-apoA-I cpm precipitated (when no unlabeled apoA-I is added to the system) minus cpm in nonspecific precipitate. T = ^{125}I-apoA-I added to tubes.

RESULTS

Homogeneity of ^{125}apoA-I

Two criteria were used to determine this homogeneity of ^{125}I-labeled apoA-I: (*a*) electrophoresis, and (*b*) immunochemistry.

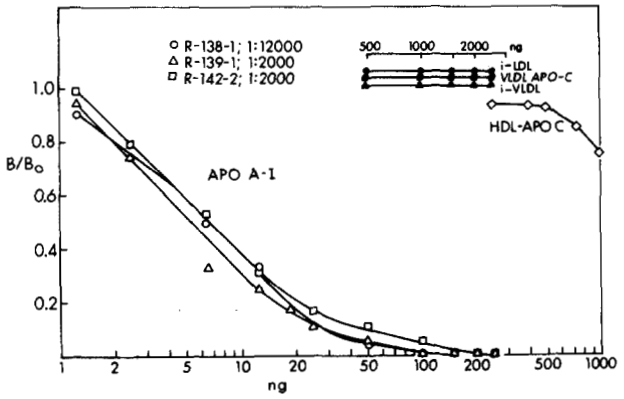

Fig. 1. Standard curves produced by the addition of increasing amounts of "cold" apoA-I to the assay. The antisera were diluted as indicated. 100 ng of apoA-I displaced nearly all of the ^{125}I-apoA-I. 500–2500 ng of rat LDL (iLDL), VLDL(iVLDL), VLDL-ApoC and the peak containing the "arginine-rich" protein displaced no counts. 750 ng of "HDL-apoC" containing both the apoC group and the apoA-II proteins of HDL produced the same amount of displacement produced by 2 ng of apoA-I.

(*a*) ^{125}I-apoA-I was subjected to electrophoresis in polyacrylamide. The gels were fixed in 10% trichloroacetic acid, sliced into 3 mm segments, and counted in a Packard Gamma Spectrometer (Packard Instrument Co., Downers Grove, Ill.). A single radioactive peak was obtained. Simultaneously, another gel containing 15 μg of apoA-I was run and stained. The radioactive peak corresponded with the stained area.

(*b*) ^{125}I-apoA-I was used in the assay. More than 90% of the label was precipitated by excess antibody. A similar percentage was precipitated by 10% trichloroacetic acid.

The assay curve

As increasing amounts of "cold" apoA-I were added, ranging from 1.25 to 250 ng, a typical radioimmunoassay displacement curve was obtained (**Fig. 1**). (Masses of apoA-I are based on the procedure of Lowry et al. (15), using BSA standards. 1 ng = 0.95 ng by amino acid analyses, thus to obtain mass units in terms of amino acid analysis, multiply given figures by 0.95.) Approximately 4 ng of protein resulted in a B/B_0 of 0.5, and virtually all of the counts were displaced by 100 ng of apoA-I. Intact LDL and intact VLDL isolated from rat plasma were added in quantities up to 2500 ng and produced no displacement. Similarly, peaks 2 and 3 isolated from VLDL by Sephadex G-200 chromatography, which contained "arginine-rich" protein and apoC respectively (11), produced no displacement in comparable amounts. Sephadex G-200 peak 3 (containing the apoC) and apoA-II isolated from HDL did produce some displacement at 750 ng. However, 2 ng of apoA-I gave a similar amount of displacement. Thus, peak 3 contained 0.3% of the activity of apoA-I. The lack of displacement by peak 3 of HDL and by peak 2 of VLDL excludes any significant reactivity of the rat apoA-II and of "arginine-rich" protein in this system. Similar results were obtained with two other antisera.

The precision of 60 points on standard curves each run in

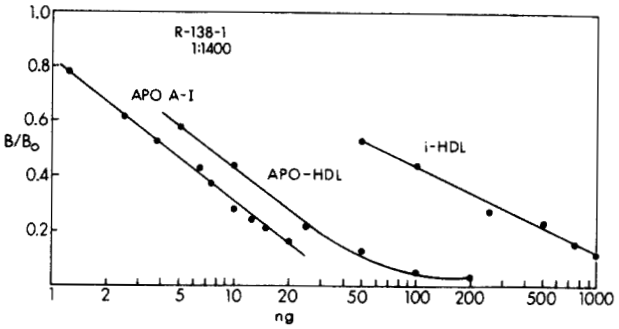

Fig. 2. Poor reactivity of intact rat HDL in assay. HDL isolated from Sprague-Dawley plasma displaced counts in parallel with apoA-I (fatty), but about 20 times as much HDL-protein was required to obtain equivalent displacement. Delipidation of HDL (apoHDL) increased its activity 10-fold, yielding an apparent apoA-I content for apoHDL of 50%.

triplicate was 7.7 ± 2.4 (coefficient of variation, mean ± 1 SD). In seven assays B_0/T was 55 ± 3%, and 4.2 ± 0.05 ng of "cold" apoA-I were required to obtain a B/B_0 of 0.5 in the same assays. The slopes of these curves averaged −2.650 ± 0.09, *y*-intercepts averaged 1.668 ± 0.076 (logit scale), and the correlation of logit B/B_0 vs. ng apoA-I averaged 0.997 ± 0.003. As little as 1.5 ng of apoA-I could be reliably detected with a precision of about 10%. ApoA-I was also isolated from Sprague-Dawley rats, and used as a standard in this assay; curves indistinguishable from the ones above were obtained.

ApoA-I in rat HDL

Approximately 16–20 times as much HDL-protein as apoA-I was required to produce a given amount of displacement (**Fig. 2**). This yields an apparent apoA-I content for HDL of approximately 5–6%. However, gel filtration and SDS disc gel data indicated that the apoA-I content of rat HDL is 45–60%. Thus, only about 10% of the apoA-I in intact rat HDL was reacting in the assay. Each of three different HDL preparations, one isolated from the fatty and two from Sprague-Dawley rats, displayed parallelism with the apoA-I standard and yielded comparable results. The delipidation of HDL with ether–ethanol solutions increased its reactivity approximately 9 ± 2-fold (mean ± 1 SD, n = 4) without altering its parallelism. Thus, delipidated HDL had apoA-I contents of 55% by RIA, close to the 45–60% obtained by column chromatography and disc gel electrophoresis.

ApoA-I content of plasma

Untreated plasmas displayed parallelism in the assay. ApoA-I levels in these plasmas were 2–7 mg/dl. When assay tubes were incubated at 37°C for two 2-hr periods, similar low results were obtained. Extracted plasmas also displayed parallelism in the assay. ApoA-I levels in these plasmas were 39 ± 8 mg/dl in Sprague-Dawley males and 47 ± 6 mg/dl in Sprague-Dawley females ($0.05 < P < 0.1$). Results were indistinguishable whether 0.1 or 0.2 ml of plasma was extracted for assay (mean difference of 10 pairs of plasmas assayed simultaneously was 0.1 mg/dl, $P > 0.5$ by paired *t* test). When the same 10 plasmas were heated to 52°C prior to assay instead of being extracted, apparent apoA-I levels were 26 ± 4 mg/dl (vs. 41 ± 6 for extracted samples). Correlation coefficients for total-chol vs. apoA-I were 0.377 ($P > 0.05$, n = 27) and for HDL-chol vs. apoA-I were 0.698 ($P < 0.001$, n = 27). HDL-chol was determined on plasmas from which VLDL and LDL had been precipitated by heparin and $MnCl_2$ (22).

DISCUSSION

Any assay must meet criteria of specificity, precision, accuracy and sensitivity before it can be deemed useful. In RIA, *specificity* is determined by the purity of the tracer (23). The rat apoA-I preparations used in these studies met several of the commonly accepted criteria of purity. In addition, iodination did not affect the reactivity of apoA-I: (*a*) ^{125}I-apoA-I preparations behaved as did "cold" apoA-I by disc gel electrophoresis. (*b*) ^{125}I-apoA-I was 90% precipitable by excess anti apoA-I, and (*c*) "cold" apoA-I competed with the tracer for limiting amounts of antibody, displacing >90% of bound ^{125}I-apoA-I (Fig. 1). The label was not displaced by preparations of rat VLDL and LDL, nor by apoC or "arginine-rich" protein isolated from VLDL. Sephadex G-200 fractions 3 isolated from HDL, which contained both apoC and apoC-II, displaced very few counts. Thus, the assay appears to be specific.

The *precision* of the assay compares favorably with previously published radioimmunoassays for human apoB (24), human apoA-I (3), and other radioimmunoassays (21). Samples are stable at 4°C for up to 12 weeks (the longest time tested). *Sensitivity* is not a limiting factor in the plasma assay since 1.5 ng of apoA-I are detectable with fair precision.

To assess *accuracy*, the apoA-I contents of HDL, as obtained by column chromatography and by SDS polyacrylamide gel electrophoresis, were compared with apoA-I contents as obtained by radioimmunoassay. The agreement between the physical and immunologic methods was remarkably good, given the limitations of the procedures involved. Thus, the content of apoA-I in HDL could be determined accurately.

The accuracy of the apoA-I levels in plasma reported here is supported by the following: (*a*) Plasma apoA-I levels obtained by radioimmunoassay were close to those one would expect from previously reported values for HDL-chol (25) and the known relationship between HDL-chol and HDL-protein (11). HDL-chol/HDL-protein and apoA-I/apoHDL ratios are each about 0.5; therefore, with HDL-chol of 31 mg/dl, HDL-protein and apoA-I should be about 60 and 30 mg/dl, respectively. The d > 1.21 fraction probably also contains a few mg/dl of apoA-I (3). The values we obtained agreed with these estimates. (*b*) The assay accurately determined the apoA-I content of HDL, which is likely to contain most of the apoA-I in plasma. (*c*) Identical results were obtained when 0.1 or 0.2 mg of plasma was extracted, suggesting that selective losses of apoA-I were not sustained during the extraction procedure.

The assay procedure would be more convenient if plasma could be assayed without prior extraction. Attempts were made to alter the temperature of incubation, i.e., to preheat the samples instead of extracting them. Fainaru, Clanglaud, and Eisenberg (20) have found the latter (preheating) to be sufficient in assaying human apoA-I. Neither of these attempts yielded acceptable results. Therefore, for the time being, extraction appears to be necessary for assaying apoA-I in plasma accurately in this system.

ACKNOWLEDGMENTS

The pioneering efforts of Barbara Pfleger and Robert Roy, and the help of Gary Grabau and Bonnie Larson are greatly appreciated.

This research was supported by grant HL 15427-02 of the National Institutes of Health, and in part by the U. S. Public Health Service contract NIH-NHLI-72-2916-L of the Lipid Research Clinics Program.

Manuscript received 3 March 1975 and in revised from 4 August 1975; accepted 26 September 1975.

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