Small HDL particles containing two apoA-I molecules are precursors in vivo to medium and large HDL particles containing three and four apoA-I molecules in nonhuman primates

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Abstract **We hypothesized that small HDL particles, containing two apoA-I molecules but no apoA-II (LpAI), may be converted in vivo into medium and large HDL particles, containing three or four apoA-I molecules, respectively, and that more conversion will occur in animals with higher HDL concentrations. To test this possibility, kinetic studies of small LpAI were performed in African green monkeys with either high plasma HDL cholesterol concentrations (120 ± 36 mg/dl, mean ± SD, n = 3) or low plasma HDL cholesterol concentrations (40 ± 13 mg/dl, n = 3). Tracer small LpAI was purified, without ultracentrifugation, by immunoaffinity and gel filtration. After injection, the specific activity of apoA-I in small, medium, and large HDL, consisting of both LpAI and LpAI:AII particles, was followed. A multicompartmental model was developed with the simultaneous analysis of urine and plasma turnover data for the kinetics of apoA-I in small, medium, and large HDL. These analyses indicated that small HDL is converted to either medium or large HDL with little or no interconversion of medium HDL and large HDL. Much of the metabolic conversion of small HDL occurs in a sequestered pool, effectively outside the circulating plasma, in a unidirectional manner before reentering the circulating plasma as medium or large HDL. The mean fractional catabolic rate of apoA-I in small, medium, and large HDL was not different comparing the high and low HDL group. In contrast, the mean production rate of apoA-I was greater in the high HDL group compared with the low HDL group. These data support the hypothesis that the plasma concentration of HDL is primarily a function of the rate of appearance of apoA-I in medium and large HDL.**—Colvin, P. L., E. Moriguchi, P. H. R. Barrett, J. S. Parks, and L. L. Rudel. **Small HDL particles containing two apoA-I molecules are precursors in vivo to medium and large HDL particles containing three and four apoA-I molecules in nonhuman primates.** ***J. Lipid Res.*** **1999.** 40: **1782–1792.**

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High density lipoprotein (HDL) is believed to protect against the premature development of complications of coronary heart disease (CHD), the leading cause of death in Western societies (1). In the many populations examined, the concentrations of plasma HDL cholesterol vary widely and those individuals with higher HDL concentrations have a lower incidence of CHD (2). The role of HDL in protecting against CHD is hypothesized to be through promotion of reverse cholesterol transport (3, 4). This is a process in which excess free cholesterol in peripheral tissues is incorporated into HDL in plasma, where it is then esterified by lecithin:cholesterol acyltransferase and transported to the liver where it can be processed. Conversion by the liver into bile acids or secretion as biliary cholesterol, with subsequent loss from the intestine, represent the major routes for cholesterol elimination from the body (5).

Accordingly, understanding the metabolism of HDL is important for clarifying the role of HDL in CHD. Earlier data from this laboratory suggested that differences in apoA-I production were related to species and diet-induced differences in plasma HDL concentration (6). The manner by which apoA-I is assimilated with lipid and HDL particles develop into the mature HDL particles found in

Abbreviations: HDL, high density lipoprotein; apo, apolipoprotein; CHD, coronary heart disease; SAAM, Simulation Analysis and Modeling; SR-B1, scavenger receptor class B type 1.

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plasma is unknown. Early work from rat liver perfusion by Hamilton and coworkers (7), as well as work from this laboratory using primate liver perfusion (8), has suggested that newly secreted HDL is present in nascent forms that require maturation in the circulation to become the variety of spherical HDL particles found in plasma (9).

In plasma, HDL is polymorphic, consisting of particles of varying size and composition that may signify distinct metabolic, functional, and clinical significance. The major apolipoproteins of HDL are thought to be important in regulating formation, stability, and metabolism of the various particles. Two classes of HDL that can be separated by sequential immunoaffinity chromatography are now recognized: those that contain only apoA-I, designated LpAI, and those that contain both apoA-I and apoA-II, designated LpAI:AII (10, 11). Both LpAI and LpAI:AII contain HDL particles across the size range of HDL (12). Studies from Nichols et al. (13, 14) and Gong et al. (15) have identified small (7.2–8.2 nm), medium (8.2–9.8 nm), and large (9.8–12.2 nm) LpAI HDL, as separated on nondenaturing gradient gel electrophoresis. Duverger et al. (16, 17) have isolated and characterized small, medium, and large LpAI particles as HDL particles with two, three, and four apoA-I molecules per particle, respectively. In general, women have more LpAI than men (17) and LpAI may be more protective against atherosclerosis than LpAI:AII (18, 19). In addition, women have more large and less medium LpAI than men (17), but the composition of large, medium, and small LpAI is similar for men and women.

The current understanding of the metabolism of HDL is derived from numerous turnover studies in which HDL apolipoproteins have been radiolabeled and reinjected (see reviews 19, 20). In almost all cases, the HDL tracer was isolated by ultracentrifugation or in some cases isolated apoA-I was reassociated with HDL particles that were previously ultracentrifuged. However, studies have shown that ultracentrifugation causes structural changes in HDL particles (12, 21) and this is a factor that may confound the interpretation of the tracer studies. Data on intact individual HDL subpopulations are needed so that we can develop an understanding of HDL metabolism that includes all aspects of HDL heterogeneity.

In our previous report (22), we examined the kinetics of large LpAI that was isolated by gel filtration and immunoaffinity chromatography without ultracentrifugation in monkeys consuming an atherogenic diet. After injection of large LpAI tracer, the specific activity of apoA-I in small, medium, and large HDL, consisting of both LpAI and LpAI:AII particles, was determined from gradient gel electrophoresis. The kinetic analysis indicated that a portion of large HDL was distributed to a noncirculating pool, outside the circulating plasma, and reentered circulating plasma after a 3-h delay after injection. There was no evidence of conversion of large LpAI to small or medium HDL particles and no evidence of transfer of free radiolabeled apoA-I from large HDL to smaller HDL particles. Although the mean fractional catabolic rate was not different, the mean production rate of apoA-I in large HDL was greater in the high HDL group compared with the low HDL group, suggesting that the plasma concentration of large HDL is controlled primarily by the rate of production of apoA-I in large HDL. Further, we found that the monkeys with higher production rates of apoA-I in large HDL also had higher plasma concentrations of apoA-I in small and medium HDL, suggesting the possibility that apoA-I in smaller HDL may be, in part, the substrate for apoA-I in large HDL.

The purpose of the present study was to test this precursor–product possibility and to determine the kinetic relationships among small, medium, and large HDL in nonhuman primates consuming an atherogenic diet in which HDL concentrations vary. We hypothesized that small LpAI HDL may be converted to medium and large HDL particles during maturation in plasma and thereby contribute to the production of large HDL particles. Alternatively, there may be direct production of large HDL from apoA-I in larger nascent HDL precursors, and smaller HDL particles from apoA-I in smaller nascent HDL precursors, as suggested from in vitro studies (14, 15), in which case the outcome would not indicate conversion of small HDL to large HDL. We report here the results of kinetic analyses of studies using small LpAI tracers isolated by gel filtration and sequential immunoaffinity chromatography. This kinetic analysis simultaneously models the turnover of apoA-I on tracer large LpAI injected at the same time but reported previously (22). The data suggest that large HDL, containing large LpAI, possibly the most atheroprotective subclass of HDL (18, 19), may be produced primarily via conversion of small HDL into large HDL.

METHODS

Animals

The six adult recipient male African green monkeys used for this study have been identified previously (22). The six recipient animals were selected from a group of 24 monkeys who were fed an atherogenic diet consisting of 35% of calories as fat (lard) and either 0.4 mg (n = 2) or 0.8 mg (n = 4) cholesterol/kcal. Two experimental groups were established with either low or high plasma HDL cholesterol concentrations (see Table 1). To maximize differences in the high and low HDL groups, recipient animals for this study were selected from both diet groups. One animal in each experimental group consumed the 0.4 mg cholesterol/kcal diet and the remaining two animals in each experimental group consumed the 0.8 mg cholesterol/kcal diet. The animals had consumed the experimental diet for at least 5 years before the metabolic studies were performed. All animals were individually housed in an enriched environment in the animal facility at the Wake Forest University School of Medicine, which is approved by the American Association for the Accreditation of Laboratory Animal Care and supervised by a veterinary staff. The Institutional Animal Care and Use Committee Approved all procedures.

Isolation of LpAI

Details of the methods used to isolate LpAI and LpAI:AII were published previously (22). Briefly, blood was drawn from the femoral vein of three donor animals. Plasma was promptly iso-

lated from the blood in the presence of preservatives and inhibitors. Fresh whole plasma was applied to an 8% agarose column and lipoproteins were separated and eluted. The distribution of the lipoproteins eluted from the column was evaluated by the measurement of total cholesterol and apolipoproteins and apoA-I-containing fractions were pooled into three different size ranges; large, medium, and small as the front, middle, and back portions of the apoA-I peak eluted from the column (22).

LpAI and LpAI:AII were isolated from the small, medium, and large HDL regions of the 8% agarose column eluate by sequential immunoaffinity chromatography using anti-apoA-I and anti-apoA-II immunoaffinity columns as previously described (22). The size-fractionated HDL samples from the 8% agarose column were applied to the anti-A-II column, the column was washed with PBS, and the bound particles were eluted with 3 m NaSCN, pH 7.4, and immediately desalted over an 80-ml Sephadex G-25 medium coarse column, equilibrated with column buffer. The eluted LpAI:AII fraction was dialyzed against column buffer and concentrated to 1 ml in Centriflo CF25 membrane cones (Amicon). The flow through from the anti-A-II column was applied to the anti-A-I column, the column was washed, and the LpAI particles were eluted, desalted, dialyzed, and concentrated as described above for the LpAI:AII particles. Aliquots of bound and unbound fractions from each column run were assayed for apoA-I and apoA-II concentrations using a sandwich enzyme-linked immunosorbent assay (23).

Lipoprotein characterization

Protein was measured by the method of Lowry et al. (24), and phospholipid was quantified by a modification of the method described by Rouser, Fleischer, and Yamamoto (25). SDS polyacrylamide gradient gel (4–30%) electrophoresis was performed as described (26). After electrophoresis, the gels were fixed and washed before autoradiography or quantification of radioactivity in gel slices. Lipoprotein particle size distribution was determined by 4–30% non-denaturing gradient gel electrophoresis as described previously (27, 28).

Iodination of LpAI

LpAI was iodinated with carrier-free ^{131}I according to the method of McFarlane (29), as modified by Bilheimer, Eisenberg, and Levy (30). Specific activities ranged from 350 to 1500 cpm/ng protein. The iodinated apoA-I-containing particles were subjected to a second size exclusion chromatography step using a 10% agarose column to obtain homogeneous small LpAI particles for reinjection. SDS-PAGE and autoradiography indicated that only apoA-I was present in the LpAI tracer with no detectable apoA-II or apoE.

In vivo metabolic studies

The reinjection studies were performed in pairs, with one recipient animal from the low HDL group and one from the high HDL group. Surgically implanted femoral artery and vein cannulas were used for blood sampling of unanesthetized jacketed and tethered animals as previously described (31). Recipient animals received oral NaI during the week before and throughout the study period to block thyroid uptake of radiolabeled iodine. On the first morning of the study, small ^{131}I-labeled LpAI (100–200 micrograms protein) was injected into the femoral vein of animals fasted overnight and 1.5-ml blood samples were collected at: 5, 10, 15, 30, 45 min; 1, 1.5, 2, 2.5, 3, 4, 6, 9, 12, 24, 36, 48, 72, 96, 120, 144, 168, and 192 h after injection. The cannulas were kept patent with a slow (~ 2 ml/h) infusion of 0.9% NaCl. On the first day of the metabolic studies, the fasted recipient animals were not fed their usual morning meal but were returned to their normal two meals per day feeding schedule with the afternoon meal. Complete daily urine samples were collected throughout the study period.

All recipient animals received tracer small LpAI isolated from three donor monkeys that were fed the 0.8 mg cholesterol/kcal diet. The small LpAI tracer was pooled from plasma collected from one donor with low concentration of HDL, one with medium concentration, and one with high. Each preparation of tracer was administered to one recipient animal from the low HDL group and one from the high HDL group. This design assured that differences observed between the low and high HDL groups are not due to differences in the composition of the administered tracer. The tracer composition might differ from the composition of HDL in circulation for each recipient animal, however we found no evidence of significant differences in composition of the small LpAI tracer and small LpAI in circulation.

Processing of plasma samples from metabolic studies

Plasma was isolated by low speed centrifugation of the timed blood samples. An aliquot of plasma was subjected to radiolabel quantification of ^{131}I using a gamma counter. Another aliquot of plasma was run on 4–30% non-denaturing gradient gels, as described above, and the regions corresponding to large (10.4–12.2 nm), medium (8.2–10.4 nm), and small (7.2–8.2 nm) and pre-beta (<7.2 nm) particles were sliced and quantified for ^{131}I radioactivity in the gamma counter. The gel slices were taken according to a template that was calibrated using the migration of Pharmacia high molecular weight protein standards and a prestained (Sudan Black) control plasma. The inter-assay coefficient of variation for radiolabel in large, medium, and small LpAI for the same plasma sample run multiple times was <5%.

A duplicate non-denaturing gradient gel of plasma samples from each recipient animal was run to determine the relative distribution of apoA-I among the large, medium, and small particles. Electrophoretic transfer and blotting with anti-apoA-I was performed as described previously (22). The stained blot was scanned with a laser densitometer and the regions corresponding to large, medium, small, and pre-beta particles (described above) were cut and weighed to calculate a fractional apoA-I distribution among the HDL particles. The fractional distribution was multiplied by the apoA-I concentration in the plasma sample, determined by ELSIA. The specific radioactivity of each size range of HDL, separated by gradient gel electrophoresis, was then calculated as cpm/mg from the cpm and mg value for apoA-I in each size fraction. The gradient gel of plasma includes apoA-II-containing particles and therefore the fractional distribution of apoA-I mass in HDL by this method includes LpAI and LpAI:AII. Therefore, the kinetic studies trace apoA-I in small, medium, and large HDL, including both LpAI and LpAI:AII.

Kinetic analysis

Analysis of the apoA-I-specific activity was completed using the SAAM II program (SAAM Institute, Seattle, WA). The simultaneous analysis of the specific activity of apoA-I in small, medium, and large HDL, and urine radioactivity data after the injection of small LpAI tracer was used to develop the HDL apoA-I kinetic model, shown in **Fig. 1**. This model incorporates our previous model of large HDL apoA-I kinetics: compartments 1 through 4 (22). The model-predicted parameters in this report were derived from the simultaneous analysis of kinetic data from small LpAI tracer and large LpAI tracer (22). During the development of the apoA-I HDL model, we examined several alternatives. Although the plasma die-away curves suggest a precursor–product relationship with small HDL converted to medium and large HDL, we first examined the hypothesis that apoA-I in small HDL may be metabolized without conversion to medium or large HDL. We found that the model of the medium and large HDL

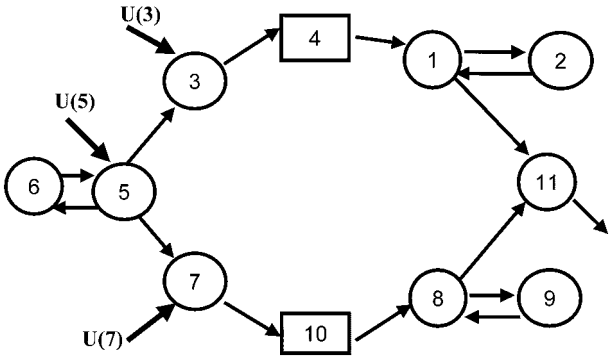

Fig. 1. Model developed from analysis of HDL apoA-I kinetic data.

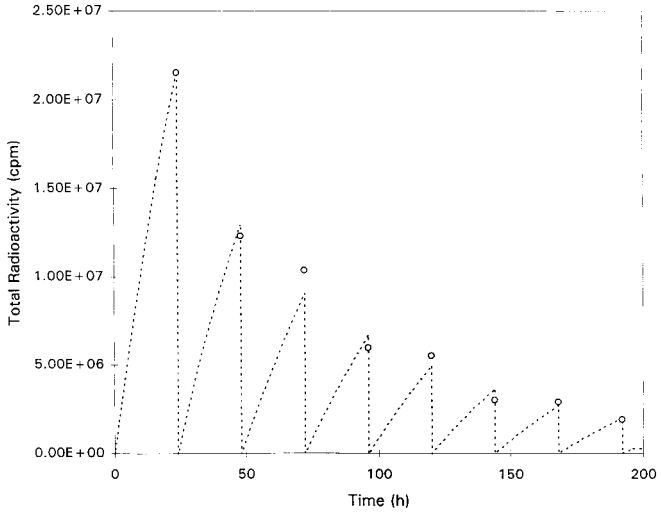

Fig. 2. Appearance of radioactivity in urine after injection of small LpAI in a representative African green monkey. (-----) Computer-predicted values; (circles) experimental data. Fits were produced from the simultaneous analysis of plasma and urine radioactivity.

data required that all of the small HDL be converted to medium or large HDL. Next, we examined the hypothesis that small HDL may be sequentially converted to medium HDL and then converted to large HDL. This preliminary model did not provide a good fit for the large HDL data during the first 12 to 24 h of the turnover studies. We then developed a model examining the hypothesis that small HDL is converted to either medium or large HDL, which provided a good fit of the kinetic data. Conversion of medium HDL to large HDL is not necessary in this model to account for the kinetic data. Further, data from a pilot study using a medium HDL tracer was consistent with the hypothesis that medium HDL is not converted to large HDL. Third, we examined the hypothesis that small HDL may be converted to medium or large HDL in the circulating plasma pool without passing through the noncirculating pool. In the model that provided the best fit of the kinetic data, all apoA-I in small HDL enters the noncirculating pool of medium or large HDL before reappearing in the circulating plasma pool as medium or large HDL.

The model that provided the best fit of all experimental data is shown in Fig. 1. This model is characterized by a circulating plasma compartment of small HDL, C(5), medium HDL, C(8), and large HDL, C(1). The circulating plasma compartments exchange with extravascular compartments, C(6), C(9), and C(2), respectively. This model includes a noncirculating compartment of medium HDL, C(7) and large HDL, C(3). The kinetic behavior of tracer apoA-I in the extravascular compartments, C(9) and C(2), is distinctly different from the kinetic behavior of tracer apoA-I in the noncirculating compartments, C(7) and C(3) and both the extravascular and noncirculating compartments are required for the model. All apoA-I in small HDL enters the noncirculating pool of medium or large HDL. ApoA-I in the noncirculating compartments, C(7) and C(3), passes through a delay compartment, C(10) and C(4), before re-entering the plasma compartment as medium or large HDL. All injected radiolabeled small LpAI is distributed among the plasma compartments, C(5), C(8) and C(1), and the noncirculating compartments, C(7) and C(3). U(5) is the production rate of apoA-I in small HDL. All apoA-I produced as small HDL is converted to medium or large HDL. U(7) and U(3) are the additional production rates of apoA-I in medium and large HDL to account for the acquisition of apoA-I molecules when small HDL particles containing two apoA-I molecules are converted to medium or large HDL particles containing three or four apoA-I molecules, respectively. The body iodine pool C(11) is assumed to turn over at a rate of 2.5 pools/d (32). Representative fit of the appearance of radioactivity in urine during a small LpAI tracer kinetic study is shown in **Fig. 2**. Representative fits of the plasma specific activity data are shown in Figs. 4–6.

In vitro incubation

The in vitro incubation studies were conducted to determine the extent to which small and large tracer LpAI can be converted to HDL particles of different size in plasma and the extent to which radiolabeled apoA-I could exchange from the tracer to HDL particles of different size in plasma. A tracer dose of large ^{125}I-labeled LpAI or small ^{131}I-labeled LpAI was incubated with undiluted whole plasma and whole plasma diluted 1:10 with PBS under N_2 atmosphere at 37°C for 0.25, 1.5, 3, and 6 h. The incubation mixture was then diluted 1:4 with PBS and an aliquot was subjected to 4–30% nondenaturing gradient gel electrophoresis for 1400 V × h at 4°C. The gel was then subjected to autoradiography to visualize the size distribution of the radiolabeled LpAI.

Data analysis and statistics

Data are reported for individual monkeys and as mean ± standard deviation for each group. A Student's *t*-test was used to determine whether significant differences in the data existed between the low and high HDL animals.

RESULTS

Six African green monkeys, that had consumed an atherogenic diet for more than five years, were selected for this study to provide two groups: a low HDL group (n = 3) with mean plasma HDL cholesterol concentration of 40 ± 13 mg/dl and a high HDL group (n = 3) with mean plasma HDL cholesterol concentration of 120 ± 36 mg/dl. Detailed analysis of plasma lipids, lipoproteins, apoproteins, and a kinetic analysis of large LpAI metabolism in these animals was published previously (22). The mean plasma concentration of apoA-I was significantly higher in the high HDL group (388.4 ± 62.9 mg/dl) compared with the low HDL group (165.7 ± 52.1 mg/dl) and higher in all subfractions of HDL with the exception of small LpAI (**Table 1**). In the high HDL group, the distri-

TABLE 1. Distribution of apoA-I in large, medium, and small HDL

Group	Low HDL	High HDL	*P*
Large LpAI	6.2 ± 3.6	47.9 ± 9.5	0.002
Large LpAI:AII	21.0 ± 9.3	48.6 ± 10.6	0.028
Large HDL	27.2 ± 11.8	96.5 ± 18.8	0.0056
Medium LpAI	14.7 ± 12.6	47.4 ± 15.8	0.048
Medium LpAI:AII	42.9 ± 13.5	91.4 ± 4.6	0.004
Medium HDL	57.6 ± 22.4	138.8 ± 12.1	0.005
Small LpAI	13.6 ± 10.2	35.7 ± 16.8	n.s.
Small LpAI:AII	67.2 ± 15.7	117.5 ± 27.6	0.052
Small HDL	80.9 ± 24.6	153.1 ± 33.9	0.041

Values for apoA-I concentration in plasma were determined by apoA-I ELISA on LpAI and LpAI:AII fractions isolated by size-exclusion and immunoaffinity chromatography. Data are the mean ± SD for each group of African green monkeys on an atherogenic diet in mg/dl; n.s., not significant.

bution of apoA-I in large, medium, and small HDL was skewed towards large HDL in that 24.9% of total apoA-I was associated with large HDL particles in the high HDL group compared with 16.1% in the low HDL group.

Kinetic studies of small HDL

The radiolabeled tracer for the kinetic studies described here was small LpAI isolated by gel filtration and immunoaffinity chromatography. **Figure 3** shows an autoradiograph of a nondenaturing gradient gel comparing three radioiodinated LpAI preparations: *1*) LpAI isolated from plasma without size-fractionation; *2*) large LpAI; and *3*) small LpAI tracer. The LpAI sample isolated from plasma without size-fractionation contains LpAI particles of a broad size range. The small LpAI tracer preparation consists primarily of small particles with no radioactivity distributed in the pre-beta (<7.2 nm) range. The tracers used for all experiments were checked with this same procedure and found to be of similar purity to those shown here. In addition, the small LpAI tracer particle size distribution was determined by 4–30% non-denaturing gradient gel electrophoresis and the distribution of radioactivity to regions corresponding to large, medium, small, and pre-beta particles was quantified. The small LpAI tracer contained 2.5% large, 8% medium, and 88.5% small LpAI particles that contained no apoA-II or apoE detectable by SDS-PAGE and autoradiography. The tracer and all plasma samples collected during the turnover study had no significant radioactivity in the pre-beta region (smaller than albumin) by gradient gel electrophoresis. Sixty percent of the injected tracer dose could be accounted for in the plasma 5 min after the injection. During the turnover study, immunoaffinity characterization of plasma from recipient animals showed that 30–40% of the injected radiolabeled LpAI tracer was rapidly converted to LpAI:AII. The percentage of radioiodinated apoA-I in LpAI:AII remained constant at 40% throughout the turnover study, suggesting that the rate of turnover of apoA-I in LpAI and LpAI:AII was similar.

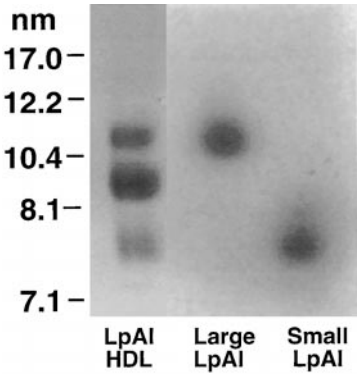

Fig. 3. Autoradiogram of nondenaturing gradient gel electrophoretograms of radioiodinated LpAI isolated from plasma, and of purified large LpAI and purified small LpAI used for injection into two animals of the study. The LpAI sample isolated from plasma without size-fractionation contains LpAI particles of a broad size range. The gradient gel was a 4–30% gradient gel prepared and run according to the procedure of Rainwater et al (38). Diameters of protein standards are indicated on the left.

In both low and high HDL groups, the specific activity of apoA-I in medium and large HDL in the 5-min plasma sample was greater than predicted from the composition of the injected small LpAI tracer and the specific activity in small HDL was less than predicted. For this reason, during the analysis of the turnover data, the distribution of the tracer to the compartments of the model was allowed to freely adjust to provide the best initial condition for each turnover study. The model-predicted initial distribution of the radiolabeled tracer (**Table 2**), or initial condition, differed from the analysis of the administered tracer dose performed at the time of the injection. In the low HDL group, the model-predicted distribution of the tracer was 8.5% to the circulating large HDL compartment, C(1), 19.5% to the circulating medium HDL compartment, C(8), and 32.1% to the small HDL compartment, C(5). The remaining 39.9% was distributed to the noncirculating compartments. In the high HDL group, the model-predicted distribution of the tracer was 9.8% to the circulating large HDL compartment, 13.5% to the circulating medium HDL compartment, and 21.8% to the small HDL compartment. The remaining 48.7% was distributed to the noncirculating compartments. Thus, although 89% of the administered tracer consisted of small HDL particles, a much smaller portion of radioactivity was

TABLE 2. Distribution of apoA-I radioactivity to compartments of HDL apoA-I kinetic model

	Low HDL	High HDL
	%	%
Circulating large HDL, C(1)	8.5	9.8
Noncirculating large HDL, C(3)	23.9	36.7
Circulating medium HDL, C(8)	19.5	13.5
Noncirculating medium HDL, C(7)	16.0	12.0
Small HDL, C(5)	32.1	21.8

Values were determined by the distribution of radioactivity in the first plasma sample collected 5 min after the injection of small LpAI tracer during the kinetic study to provide the initial condition during the kinetic modelling of the experimental data. Data are the best fit determined for each group of African green monkeys. Compartments C(1), C(3), C(5), C(7), and C(8) refer to the model shown in Fig. 1.

accounted for as small HDL particles in the 5-min plasma sample. These observations suggest that small LpAI tracer was distributed to both the circulating plasma and a noncirculating pool. This hypothesis was supported during the analysis by the lack of evidence that a portion of the small LpAI tracer was rapidly removed from plasma and catabolized. Furthermore, the tracer initially distributed to the noncirculating compartments returns to the plasma compartment later during the turnover study as medium and large HDL (described below).

During the kinetic study, the plasma die-away curve of apoA-I specific activity in small HDL was a biphasic curve in all recipients. An increase in the specific activity of apoA-I in medium and large HDL was observed following the rapid initial decrease of small HDL specific activity. As described in the Methods section, several hypotheses were examined during the development of the apoA-I HDL model. In the model that provided the best fit of the kinetic data, small HDL was not sequentially converted to medium HDL and then converted to large HDL. On the contrary, the experimental data and the kinetic model provide evidence for unidirectional conversion of small HDL to medium or large HDL. Conversion of medium HDL to large HDL was not necessary in the model to account for the experimental data. Consistent with our earlier observations of the kinetics of large LpAI, there was no evidence of conversion of medium or large HDL to smaller HDL particles in this turnover study of small LpAI tracer.

The experimental data suggest that small HDL is not converted to medium HDL or large HDL in the circulating pool. The peak in specific activity of apoA-I in medium and large HDL was not reached until several hours after the specific activity curves of medium and large HDL crossed the initial component of the small HDL die-away curve, suggesting that there is a time delay during the conversion of small HDL to medium or large HDL. In the model that provides the best fit of the data, apoA-I in small HDL enters the noncirculating pools of medium or large HDL before reappearing in the circulating pool as medium or large HDL. The delay time (2.99 ± 1.15 h) or time from the disappearance of radiolabeled small HDL apoA-I from the circulating pool to its reappearance in the circulating pools as medium or large HDL was equal to the delay time of the noncirculating compartment (2.99 ± 1.15 h) we observed during kinetic studies of large LpAI reported previously in these monkeys (22). As in our previous report, all injected tracer radioactivity was accounted for in the model. There was no evidence that the delayed peak was the result of partial extravasation of tracer into subcutaneous or extravascular tissue during the injection of the tracer dose which might produce a delayed leakage of tracer material into plasma after the start of the turnover study. There also was no evidence that the delay was an artifact of the re-isolation of large, medium, and small HDL from plasma samples collected during the turnover study or due to a change in plasma concentration of apoA-I during the turnover study.

The HDL apoA-I kinetic model-predicted values (represented by the lines) are consistent with the plasma experimental data (represented by the data points) for apoA-I specific activity in large, medium, and small HDL as seen in **Fig. 4** for a representative monkey from the high HDL group. After the injection of radiolabeled small LpAI, the specific activity of apoA-I in small HDL rapidly decreased and, after a delay, a peak in the specific activity of apoA-I in both medium and large HDL was observed. Figures 5 and 6 compare the HDL apoA-I kinetic model-predicted values to the plasma experimental data in two monkeys from the low HDL group that represent the two patterns observed in the low HDL group. After the injection of radiolabeled small LpAI in the turnover study, shown in **Fig. 5**, the apoA-I specific activity increased predominantly in large HDL, whereas in **Fig. 6** the apoA-I specific activity increased predominantly in medium HDL. Although the experimental kinetic data provide clear evidence of the transfer of tracer radioactivity from small HDL to medium or large HDL during the turnover study in all monkeys

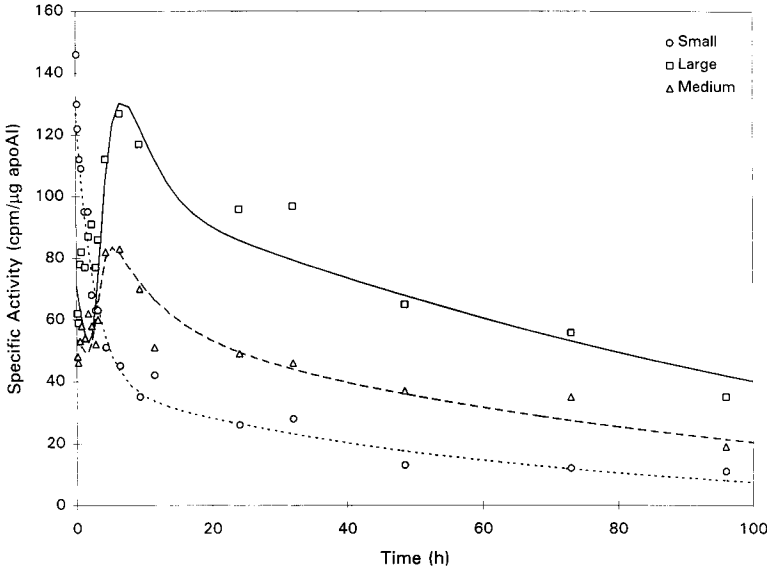

Fig. 4. Specific activity for apoA-I in large (squares), medium (triangles), and small HDL (circles) after the injection of small LpAI in a representative African green monkey from the high HDL group. Lines are the computer-predicted values. Experimental data is specific activity expressed as CPM/microgram apoA-I.

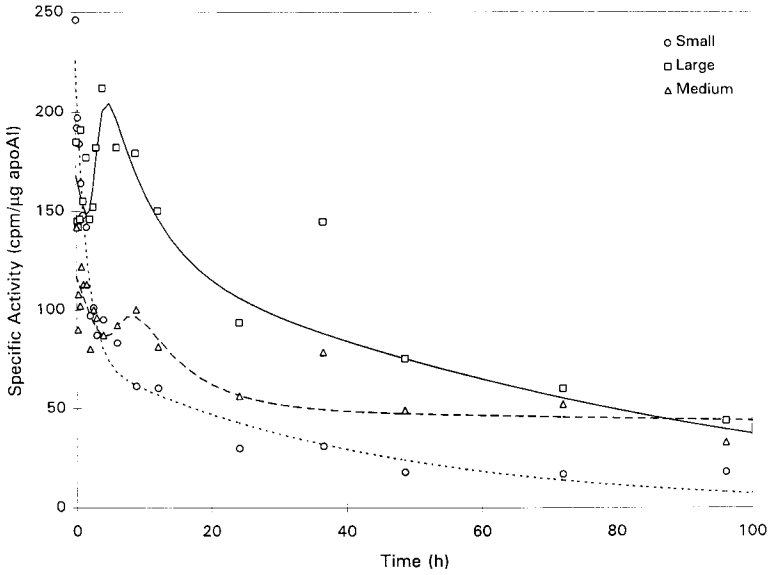

Fig. 5. Specific activity for apoA-I in large (squares), medium (triangles), and small HDL (circles) after the injection of small LpAI in an African green monkey from the low HDL group. The apoA-I specific activity was increased predominantly in large HDL in this monkey. Lines are the computer-predicted values. Experimental data is specific activity expressed as CPM/microgram apoA-I.

studied, there was no evidence of significant transfer of tracer radioactivity from large HDL to medium or small HDL, nor from medium HDL to large HDL, and no evidence of any transfer of tracer radioactivity to apoB containing lipoproteins or pre-beta HDL.

Small and large LpAI tracers were simultaneously administered to each animal and the data were modeled simultaneously. The kinetic behavior of large HDL tracer that was directly injected was the same as the kinetic behavior of the large HDL that was derived from the small HDL tracer. The simultaneous analysis of the plasma specific activity, including small, medium, and large HDL, and urine radioactivity was used in the HDL apoA-I model to characterize the interconversion among the three subpopulations of HDL and to calculate the fractional catabolic rate and production rate for each. The model-predicted fractional catabolic rates and production rates are shown in **Table 3**. There was no significant difference in the mean fractional catabolic rate of apoA-I in small, medium, and large HDL comparing the low and high HDL groups. The increased plasma concentration of apoA-I in the high HDL group was attributable to a significantly greater total production rate of apoA-I in this group of monkeys. The production rate of apoA-I in small, medium, and large HDL was significantly greater in the high HDL group compared with the low HDL group. All apoA-I in small HDL was converted to medium or large HDL. In the high HDL group, 55% of the apoA-I in small HDL was converted to medium HDL and 45% was converted to large HDL, whereas in the low HDL group 84% was converted to medium HDL and 16% was converted to large HDL. The total production rate of apoA-I in medium and large HDL includes the apoA-I derived from the conversion of small HDL, containing two apoA-I molecules per particle, as well as additional apoA-I that was not derived from the labeled small HDL to account for the acquisition of apoA-I

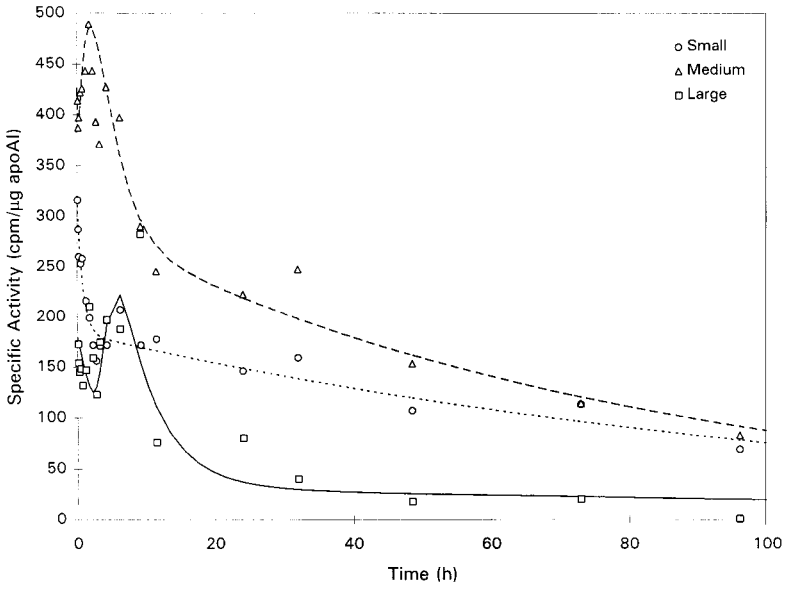

Fig. 6. Specific activity for apoA-I in large (squares), medium (triangles), and small HDL (circles) after the injection of small LpAI in an African green monkey from the low HDL group. The apoA-I specific activity was increased predominantly in medium HDL in this monkey. Lines are the computer-predicted values. Experimental data is specific activity expressed as CPM/microgram apoA-I.

TABLE 3. Model-predicted metabolic parameters of HDL apoA-I after injection of radiolabeled small LpAI

	Low HDL	High HDL
Fractional catabolic rate (pools/day)		
ApoA-I in small HDL	0.559 ± 0.13	1.027 ± 0.59
ApoA-I in medium HDL	1.054 ± 0.38	0.715 ± 0.22
ApoA-I large HDL	0.414 ± 0.47	0.855 ± 0.17
Production rate (mg apoA-I/h)		
ApoA-I in small HDL	18.4 ± 4.6	49.1 ± 7.2[a]
ApoA-I in medium HDL		
Derived from small HDL	15.3 ± 2.9	27.1 ± 6.1[b]
Independent of small HDL tracer	7.7 ± 1.5	13.6 ± 3.1[b]
Total medium	23.0 ± 4.5	40.7 ± 9.2[b]
ApoA-I in large HDL		
Derived from small HDL	3.0 ± 3.7	21.9 ± 9.4[c]
Independent of small HDL tracer	3.0 ± 3.7	21.9 ± 9.4[c]
Total large	6.1 ± 7.4	43.9 ± 18.8[c]
Total apoA-I production	29.1 ± 8.5	84.6 ± 14.7[d]

Data are the mean ± SD of the model-predicted fractional catabolic rate and production rate of apoA-I for each group of African green monkeys consuming the experimental diet. No difference was found comparing the fractional catabolic rate of apoA-I in small, medium, and large HDL between the low and high HDL groups. All apoA-I produced in small HDL is converted to medium or large HDL. The total production rate of apoA-I in medium HDL includes two apoA-I molecules derived from the conversion of small HDL particles plus the addition of one apoA-I molecule for each medium HDL particle, containing three apoA-I molecules, produced. The total production rate of apoA-I in large HDL includes two apoA-I molecules derived from the conversion of small HDL particles plus the addition of two apoA-I molecules for each large HDL particle, containing four apoA-I molecules, produced. Mean production rate of apoA-I is significantly increased in the high HDL group.

[a] $P = 0.003$.
[b] $P = 0.040$.
[c] $P = 0.032$.
[d] $P = 0.0048$.

molecules by medium HDL particles, containing three apoA-I molecules, and large HDL particles, containing four apoA-I molecules. Thus, the mean total production rate of apoA-I was greater in the high HDL group, which was accounted for by increased mean production rates of apoA-I in small, medium, and large HDL.

In vitro conversion of HDL subfraction

To determine the extent to which interconversions of small and large LpAI could occur in vitro or the extent to which radiolabeled apoA-I could exchange from the tracer LpAI to other HDL particles of differing size, we conducted experiments in which small or large ^{125}I-labeled LpAI were incubated with undiluted whole plasma or diluted whole plasma for up to 6 h at 37°C. **Figure 7** shows a representative autoradiogram of a 4–30% non-denaturing gradient gel separation of plasma LpAI after different lengths of incubation. Little in vitro transfer of radioactivity from small to large HDL or large to small HDL was observed under these conditions.

DISCUSSION

In cholesterol-fed African green monkeys, as is the case in human beings, high plasma HDL cholesterol concentrations have been shown to be inversely correlated with coronary artery atherosclerosis (33). In the group of cholesterol-fed African green monkeys of this study, higher HDL cholesterol concentrations were associated with higher total plasma concentrations of apoA-I and increased concentrations of apoA-I in medium and large HDL. This increase in the plasma concentration of apoA-I was attributable to increased production of apoA-I and increased conversion of apoA-I in small HDL to medium or large HDL. In our previous study using large LpAI tracer, the data suggested that large HDL particles were removed directly from plasma without conversion to small or medium particles and by implication that small HDL might be the precursor to large HDL (22). The results of the present study are consistent with these observations and extend our findings to small and medium HDL. These tracer studies are the first, to our knowledge, to clearly demonstrate that small HDL are converted in vivo, in a unidirectional manner to medium or large HDL. Medium HDL behaved in a manner kinetically similar to that of large HDL, i.e., as stable particles removed directly from plasma without conversion to small or large particles. Thus, taken together, these studies suggest that medium and large HDL may be end-products in the HDL metabolic pathway. The rate of production of medium and large HDL may possibly be a measure of the capacity for reverse cholesterol transport in an individual.

These data show several departures from commonly assumed behaviors of apoA-I in the circulation. First, the random exchange among HDL subfractions of free radiolabeled apoA-I was minimal; rather, a directional movement of apoA-I was observed. We suspect that this difference is because the HDL tracer was not isolated by ultracentrifugation in our experiments. Second, little or no appearance of radiolabeled apoA-I in a small or preβ HDL fraction was observed during these turnover studies. We suspect that this means that the bulk of the preβ mate-

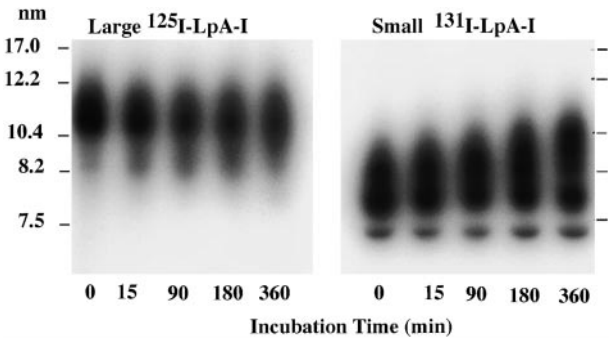

Fig. 7. A tracer amount of large ^{125}I-labeled LpAI or small ^{131}I-labeled LpAI was incubated with whole plasma diluted 1:10 with phosphate-buffered saline under N_2 atmosphere at 37°C for the indicated times. The incubation mixture was then diluted 1:4 with phosphate-buffered saline and an aliquot was subjected to 4–30% nondenaturing gradient gel electrophoresis for 1400 V × h at 4°C. The gels were exposed to film to visualize the radiolabeled LpAI. Migration position of the molecular weight standards is shown for reference.

rial commonly found in plasma is derived from newly secreted apoA-I that is in the process of being converted into mature HDL particles. It is also possible that some apoA-I in the small, lipid-poor preβ fractions might be derived from the remodeling of larger HDL but was not observed during these studies because it is rapidly cleared in vivo. The kinetic analysis suggests that this was not a major pathway. However, more kinetic studies using tracers that have not been isolated by ultracentrifugation should help identify the best interpretation. Third, the rapid sequestration and subsequent return of HDL particles to the plasma, which was a prominent feature of these data, has not previously been highlighted in turnover studies. The sequestration may represent the interaction of HDL particles with proteins at cell surfaces, such as the putative Tangier's protein (34), SR-B1 (35), and maybe even phospholipid transfer protein (36), either in endothelial cells lining the plasma compartment or on cells lining interstitial spaces into which HDL particles filter (37). The most efficient sequestration may require a specific conformation of apoA-I that might be preserved during immunoaffinity isolation but disrupted during ultracentrifugation. The rapid distribution of LpAI tracer after re-injection has not been reported in stable isotope studies primarily due to the complex precursor kinetics of the amino acid being traced.

The available data in humans (17) suggests that individuals with different HDL concentrations have shifts in HDL subfraction distribution but not differences in the composition of HDL subfractions. Although currently there is no evidence that differences exist in the composition of small HDL isolated from animals with low HDL concentration versus animals with high HDL concentration, to control for potential differences that might exist, the tracer in this study was small LpAI derived from pooled plasma from donor animals with a range of plasm HDL cholesterol concentration. Thus, comparison of tracer catabolism in the low HDL group versus the high HDL group can be made while reducing any potential confounding effect of differences in the tracer used in the two groups.

This study demonstrates that small LpAI can be isolated by gel filtration and sequential immunoaffinity chromatography and radiolabeled for reinjection studies while maintaining the integrity of the tracer. Several previous studies using HDL tracers that were isolated by ultracentrifugation have described a subpopulation of apoA-I that is 'loosely' associated with the HDL tracer and rapidly transfers from the HDL tracer to other lipoproteins in plasma after reinjection. In our previous study using large LpAI tracer isolated without ultracentrifugation, we found that the radiolabeled apoA-I tracer remained associated with large HDL throughout the turnover study with no evidence of significant transfer of apoA-I from large HDL tracer to other lipoproteins. In contrast, in the present study there was evidence of remodeling of a small amount of small LpAI tracer to medium and large HDL during the first 5 min after reinjection, but no evidence of nonspecific transfer of radiolabeled free apoA-I from the tracer to other lipoproteins. We do not believe that nonspecific exchange of apoA-I is a primary process in HDL metabolism for the following reasons. *1*) There was no evidence of exchangeable apoA-I in the large LpAI tracer study (22); *2*) the in vitro incubation study revealed no evidence of exchange of apoA-I from small LpAI to other lipoproteins of differing size; and *3*) there was only movement of apoA-I from small to larger HDL during the turnover study. We suspect that small LpAI tracer particles are remodeled and large HDL tracer particles are not because small HDL particles are the precursors of larger HDL particles.

As in our previous report of the kinetics of large LpAI tracer, 40% of the injected small LpAI tracer was converted to LpAI:AII particles early during the turnover study. Rader et al. (38) previously reported that radiolabeled apoA-I on LpAI tracers could be recovered from LpAI:AII particles after re-injection. Because the preponderance of evidence, discussed above, suggests that apoA-I is not randomly exchanged between HDL particles, we suspect that the conversion of LpAI tracer to LpAI:AII particles may be the result of the gain of an apoA-II molecule(s) by the tracer. However this study was not designed to directly examine this process.

The existence of the noncirculating compartments, C(3) and C(7), and other extravascular compartments, C(2), C(9), and C(6), as separate compartments is based on the kinetic behavior of tracer LpAI distributed to these compartments. Forty percent of the small LpAI tracer was distributed to the noncirculating compartment at the start of the turnover study and returned to the circulating plasma after a delay. The delay time is a measure of the average time a small HDL particle will spend in the noncirculating pool before appearing in the plasma as a medium or large HDL particle. The delayed return of tracer from the noncirculating compartment produced the delayed peak in specific activity observed in medium and large HDL. Excluding the rapid remodeling of the tracer small HDL discussed above, the conversion of small HDL to larger HDL particles occurred in the noncirculating compartment. The results of the in vitro incubation studies of radiolabeled small and large LpAI support this hypothesis, that conversion of small HDL to larger HDL occurs in a noncirculating compartment, because during the incubation study serum alone was not sufficient for the conversion of small LpAI tracer to medium and large HDL. Furthermore, during modeling of the turnover data, we examined the hypothesis that small HDL could be converted to larger HDL without entering the noncirculating compartment. This hypothesis was not found to be consistent with the experimental data. These observations are consistent with the hypothesis that the conversion from small HDL to medium and large HDL occurs in a sequestered, extravascular pool.

In our previous study using tracer large LpAI, we hypothesized that the noncirculating compartment represented HDL particles sequestered from the circulating plasma in the margins of peripheral vessels, within the porous pathways of the vascular endothelium or in the subendothelial spaces of arteries, for example. While distributed to these anatomical locations, HDL particles

would be in close physical proximity with cells and would not be detected in samples of plasma collected during the kinetic study. Small HDL particles in close physical proximity with cells may bind to cell surface receptors, such as the putative Tangier's protein or SR-B1, and could accept the transfer of cellular cholesterol and/or phospholipid (34, 35), a process that would delay the return of maturing HDL particles to the circulating compartment. The results of the current kinetic studies are also consistent with this hypothesis. However, the kinetic data in this study are limited to the turnover of apoA-I and the rate of movement of cholesterol through HDL was not measured. The data support the assumption that HDL particles are cleared from the plasma as an intact entity. Although cholesterol turnover was not directly examined, the observations made during these kinetic studies suggest that small HDL particles are distributed to physical spaces that would allow the uptake of excess cellular cholesterol and the return of the HDL particle to the circulating plasma as a larger particle that has gained apoA-I, cholesterol, and phospholipid.

The kinetic model developed during this study simultaneously models the specific activity of apoA-I achieved in small, medium, and large HDL, the volume of distribution and pool size of each, and the accumulation of radiolabel in urine. The experimental data and the kinetic model suggest that all small HDL is converted to medium or large HDL before catabolism and that a precursor of apoA-I in medium and large HDL include, but may not be limited to, apoA-I in small HDL. The model-predicted production rates for apoA-I in medium and large HDL include both apoA-I derived from the conversion of small HDL plus an additional source of apoA-I to account for the gain of apoA-I molecules by small HDL particles during maturation. For instance, the mean total production rate of apoA-I in large HDL, containing four apoA-I molecules per particle, in the high HDL group is 43.9 $\pm$ 18.8 mg apoA-I/h. Of this total, 21.9 $\pm$ 9.4 mg apoA-I/h is derived from small HDL, containing two apoA-I molecules per particle, and 21.9 $\pm$ 9.4 mg apoA-I/h is derived from an additional source of apoA-I. This additional source of apoA-I in medium and large HDL was increased in the high HDL group and may represent direct hepatic production of apoA-I in medium and large HDL.

The process by which small HDL particles acquire additional apoA-I molecules and cholesterol during maturation to larger HDL particles is only a matter for speculation. Nichols and colleagues (13–15) have studied in vitro transformations of recombinant HDL and some of these modifications may occur in vivo. However, we have injected only spherical HDL and the applicability of LCAT-mediated transformations studied by these workers to the conversions documented here is unknown. Small HDL particles may combine with nascent particles to become larger HDL particles or small HDL particles may mature by the gradual addition of constituents. The process of maturation of small HDL appears to be unidirectional but the amount of final product, medium HDL or large HDL, varied among animals and between groups. In the low HDL group, apoA-I in small LpAI was converted predominantly to medium HDL. In the high HDL group, apoA-I in small HDL was converted more equally to both medium and large HDL. At present, there is insufficient evidence to explain the basis for this difference but the possibility that more small HDL is available for maturation in the high HDL group is a likely factor.

In this group of African green monkeys fed moderately atherogenic diets, higher HDL cholesterol concentration was associated with higher concentrations of apoA-I predominantly in medium and large HDL. This study, we believe, is the first to demonstrate the conversion of small HDL, in a unidirectional manner, to medium or large HDL. This conversion appears to occur in a noncirculating pool, outside the circulating plasma, that we hypothesize to be in close physical proximity with cells that would allow the transfer of excess cellular cholesterol to maturing small HDL particles during reverse cholesterol transport. The increased plasma concentration of HDL in cholesterol-fed African green monkeys, which confers decreased risk for CHD, was attributable to a significant increase in the production rate of apoA-I and increased conversion of small HDL to larger HDL, but not to a decrease in the fractional catabolic rate. This observation, that plasma HDL concentration is determined by production of apoA-I and not the fractional catabolic rate in cholesterol-fed African green monkeys, might not be applicable to monkeys consuming a chow diet. ApoA-I in medium and large HDL is derived from the conversion of small HDL plus an additional source of apoA-I that may represent direct hepatic production of apoA-I in medium and large HDL.

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