Tissue Sites of Degradation of High Density Lipoprotein Apolipoprotein A-IV in Rats

Gees M. Dallinga-Thie, Ferdinand M. Van 't Hooft, and Arie Van Tol

The in vivo metabolism of high density lipoprotein (HDL), labeled by incorporation of $^{125}$I-apolipoprotein (apo) A-IV, was studied in the rat and compared with the metabolism of HDL labeled with $^{131}$I-apo A-I. The $^{125}$I-apo A-IV labeled HDL was obtained by adding small amounts of radioiodinated apo A-IV to rat serum, followed by separation of the different lipoprotein fractions by chromatography on 6% agarose columns in order to avoid "striping" of apolipoproteins by ultracentrifugation. Under both in vitro and in vivo conditions, the $^{125}$I-apo A-IV remained an integral component of HDL and was not exchanged to other lipoproteins, including the "free" apo A-IV fraction. The serum half-life, measured at between 8 and 28 hours after intravenous injection of labeled HDL, was 8.5 ± 0.5 hours for HDL apo A-IV and 10.2 ± 0.7 hours for HDL apo A-I. The tissue sites of catabolism of HDL apo A-IV and HDL apo A-I were analyzed in the "leupeptin-model." Only the kidneys and liver showed a significant leupeptin-dependent accumulation of radioactivity. At 4 hours after injection of $^{125}$I-apo A-IV/$^{131}$I-apo A-I labeled HDL, 3.5% ± 1.0% and 8.4% ± 2.0% of HDL apo A-IV and 4.6% ± 1.3% and 2.6% ± 0.6% of the HDL apo A-I were accumulated in a leupeptin-dependent process in the kidneys and liver, respectively. Immunocytochemical studies revealed that the renal localization of apo A-IV was intracellular and confined to the epithelial cells of the proximal tubuli. The amount of intracellular apo A-IV in rat kidneys was increased in leupeptin-treated animals. The data suggest that the leupeptin-dependent degradation of HDL apo A-IV is more active in the liver than in the kidneys, while the opposite was observed for HDL apo A-I. These results, as well as the short half-life of HDL apo A-IV as compared to HDL apo A-I, are compatible with the existence of an apo A-IV-containing HDL subfraction with a relatively fast turnover for which the liver is the major catabolic site. (Arteriosclerosis 6:277–284, May/June 1986)

Considerable evidence in favor of a positive correlation between low levels of high density lipoprotein (HDL) cholesterol and an increased risk of coronary heart disease and atherosclerosis has been obtained from epidemiological studies. However, little is known about the exact physiological function of HDL. Glomset postulated in 1968 that HDL may serve to transport cholesterol from the extrahepatic tissues to the liver ("reverse cholesterol transport"). Since then, several studies showed that in the rat HDL can deliver cholesterol(ester) to the liver and to organs that are involved in the synthesis of steroid hormones.

Serum apolipoprotein (apo) A-IV is found primarily in HDL and in a "free" apo A-IV fraction. Turnover studies in the rat showed that the half-life of iodinated HDL-bound apo A-IV in the circulation is not different from that of total iodinated HDL. This study suggested that, upon leaving the lymph compartment and entering the plasma compartment, apo A-IV was transferred to the HDL fraction by way of the "lipoprotein-free" fraction. It has been shown that this transfer of apo A-IV from the "free" fraction to HDL is due to the esterification of free cholesterol by lecithin:cholesterol acyl transferase.

Recent studies on the catabolic sites of other HDL apoproteins, apo A-I and apo E, showed that the kidneys play an important role in the degradation of apo A-I, as well as apo E. In the present paper, we studied the degradation sites of HDL apo A-IV, using leupeptin-treated rats. Our study shows that the kidneys contribute significantly to the degradation of HDL apo A-IV, although the liver is the major catabolic site.

Methods

Materials

Sodium $^{125}$I-iodide (carrier-free, 350–600 mCi/ml) and sodium $^{131}$I-iodide (carrier-free, 40 mCi/ml) were obtained from Amersham International, Amersham, Buckinghamshire, Great Britain. Leupeptin was obtained from the Peptide Institute, Osaka, Japan. Agarose (6%) was obtained from Bio Rad, Richmond, Virginia, and swine antirabbit
serum immunoglobulin, conjugated to horse-radish peroxidase, was purchased from Dako, Glostrup, Denmark. Rat serum albumin and diaminobenzidine were obtained from Sigma Chemical Company, St. Louis, Missouri.

**Treatment of Rats**

Male Wistar rats weighing 350 to 400 g were maintained on standard laboratory chow and tap water. Where indicated, food was withheld for 20 hours. Blood was collected from the animals by aortic puncture under light ether anesthesia. The blood was kept on ice for 2 hours, and serum was obtained by low-speed centrifugation at 4° C. These procedures followed institutional guidelines for animal research.

**Isolation and Iodination of Apo A-IV and Apo A-I**

Apo A-IV and apo A-I were isolated from rat serum HDL as described previously. After the pure apolipoprotein fractions were dialyzed extensively against 0.05 M Na-phosphate (pH 7.8) and were stored at −80° C. The purity of apo A-IV and apo A-I was checked by gelelectrophoresis on 12.5% polyacrylamide, containing 0.1% SDS, as described by Cleveland et al.

Radioiodination of apolipoproteins was carried out by using the ICI method. In short, 0.03 mg of apo A-I or apo A-IV, in a volume of 0.03 ml, was mixed with 0.02 ml 0.1 M glycine-NaOH buffer (pH 10.0), 0.01 ml 125I or 131I (0.5 mCi), and 0.005 ml ICI solution in 2 M NaCl (3.1 or 3.8 mmol for the iodination of apo A-IV and apo A-I, respectively), followed by incubation at room temperature for 5 minutes. Unreacted 125I or 131I was removed by chromatography on Sephadex G-50 (medium), equilibrated with 2 mM Na-phosphate (pH 7.4), 0.01% NaN3, and 1% gelatin, containing 2 mM Na-phosphate (pH 7.4) and 0.01% NaN3, and 1 mM EDTA. The flow rate was 3 to 4 ml/hour and fractions of 1 ml were collected. The column was operated at 4° C. Absorbance at 280 nm, 125I- and 131I-radioactivity, and total cholesterol were measured in each fraction. HDL fractions containing 125I-apo A-IV and 131I-apo A-I were pooled and dialedyzed for 18 to 24 hours against Krebs-Henseleit buffer. All preparations were immediately used for metabolic studies.

**Metabolic Studies**

After the chow-fed, nonfasted animals were anesthetized with diethylether, 1 ml of 125I-apo A-IV/131I-apo A-I-labeled HDL was injected through a femoral vein. Leupeptin was administered intravenously, as described by Van't Hooft et al., 1 hour before the injection of labeled HDL. The rats used for the 4-hour decay time-point received a second leupeptin injection 2.5 hours after the first one. Leupeptin was injected in a concentration of 20 mg/kg body weight. All control animals were handled identically, but they received injections of saline instead of leupeptin. At the end of the experiments, the rats were bled from the abdominal aorta. The heart, liver, spleen, kidneys, testes, adrenals, and representative sections of muscle, adipose tissue, lung, skin, and jejunum were removed. Calculations of the radioactivity present in the organs were made as described previously. The radioactivity in serum was measured in 0.25 ml samples, and the results were expressed as percentages of the injected radiolabel based on a plasma volume of 3.36% to 3.51% of body weight, dependent on body weight.

For “screening,” double-labeled HDL was injected into the femoral vein of chow-fed animals. The blood was collected after 1 hour and kept on ice for 2 hours. The serum was isolated and used directly for the in vivo experiments.

The label and mass distribution of apo A-IV and apo A-I were measured in the serum of rats injected with labeled HDL. Serum obtained from control or leupeptin-treated animals was subjected to 6% agarose column chromatography (column size, 2 × 120 cm) to separate the different apo A-IV-containing lipoprotein fractions. In all samples, 125I- and 131I-radioactivity (recoveries 88% ± 3% and 85% ± 5%, respectively), apo A-IV and apo A-I mass (recoveries 99% ± 1% and 98% ± 4%), respectively) and total cholesterol (recovery 96% ± 4%) were determined.

**Immunocytochemical Methods**

The immunocytochemical localization of apo A-IV in rat kidneys was performed as described by Van Ewijk et al. After the kidneys had been perfused in situ with 0.9% NaCl, containing 1 mM EDTA (pH 7.4) at 37° C, they were removed from the leupeptin-treated and control rats and embedded immediately in Tissue-tek on specimen stubs frozen on solid carbon dioxide. Frozen sections (5 μm) were cut on a cryostat (Bright Limited, Huntingdon, Great Britain) and were collected on microscope slides precoated with a solution containing 0.1% gelatin and 0.01% chromium-potassium sulphate. The tissue was gently fixed by dipping the slide for 1 second in acetone. The sections were stored at −20° C. All subsequent procedures were performed at room temperature. Before being incubated with antisera, the sections were soaked in 0.9% NaCl (containing 10 mM Na-phosphate (pH 7.4), 5% bovine serum albumin (PBS-BSA), and 0.05% Tween-20) for 30 minutes in order to remove the embedding medium. Next, the sections were covered with 50 μl of the antibody (1:300 dilution) and incubated for 1 hour in a moist chamber. After rinsing with PBS-BSA buffer, the sections were incubated for 1 hour with 50 μl swine antirabbit serum immunoglobulin conjugated to horse-radish peroxidase (1:40 dilution, containing 1% rat serum). After rinsing in PBS-BSA, the conjugate was visualized by incubation of the sections in diaminobenzidine (DAB) according to the method of Graham et al. The sections were subsequently incubated in a solution of 0.5% chromium sulphate in 0.9% NaCl to

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improve the staining. After dehydration and fixation, the sections were cover-slipped and then examined with a microscope.

Chemical Analysis

The total cholesterol was measured enzymatically by using the CHOD-PAP kit (Boehringer, Mannheim, FRG, Cat. No. 310328). Apo A-I and apo A-IV were determined by electroimmunoassay, as described previously. Iodine radioactivity was counted in the LKB-Wallac ultrogamma counter (LKB-Wallac, Turku, Finland). The data collected in this study were statistically evaluated by using Student’s unpaired t test. Values given are means ± SD for three to six experiments.

Results

Radioiodinated apo A-IV and apo A-I were incorporated into rat serum lipoproteins, and the different lipoprotein fractions were separated on a 6% agarose column. Figure 1 shows the in vitro incorporation of 125$I$-apo A-IV into serum lipoproteins, by use of a method described for the labeling of rat apo A-I and apo E. More than 80% of the label was on particles with the size of typical HDL. Fractions containing the highest level of radioactivity and 90% of total HDL cholesterol (indicated by the horizontal bar) were pooled and immediately used for metabolic studies. In all studies, double-labeled apo A-IV/apo A-I HDL preparations were used, which contained 125$I$-apo A-IV and 131$I$-apo A-I or vice versa.

In control experiments, we tested whether the labeled HDL apolipoproteins remained associated with the HDL fraction under various in vivo and in vitro conditions (see below). After rechromatography of the HDL, labeled apo A-IV and apo A-I remained quantitatively associated with the HDL fraction (not shown). Figure 2 A shows the elution pattern and the distribution of radioactivity on a 6% agarose column of pooled sera obtained from two saline-treated rats killed 4 hours after injection of HDL labeled with 125$I$-apo A-IV and 131$I$-apo A-I.

Figure 2 B shows the mass distributions of apo A-IV and apo A-I in the same experiment. In rat serum, apo A-IV was present in HDL as well as in a “free” apo A-IV fraction. In this “free” fraction, 33% ± 3% of the mass of apo A-IV was present, but 125$I$-apo A-IV could not be detected. The radioactive apolipoproteins were present on HDL particles with the same size as the nonradioactive apolipoproteins. Apo A-IV was present on slightly bigger particles than apo A-I in all sera tested. Serum obtained 3 minutes or 1 hour after injection of the labeled HDL gave essentially the same results as those shown in Figure 2 at higher levels of HDL radioactivity.

These data suggest that 125$I$-apo A-IV, once associated with HDL, will not exchange or transfer to other lipoprotein
Figure 3. In vitro incubation of $^{125}$I-apo A-IV/$^{131}$I-apo A-I HDL with serum obtained from chow-fed rats followed by chromatography on 6% agarose (column size 2 x 120 cm). The distributions of $^{125}$I-apo A-IV (•), $^{131}$I-apo A-I (○), and total cholesterol (△) are shown after 0 hours (A) and 4 hours (B) of incubation of 37° C. VLDL, LDL, HDL, RSA indicate the elution volumes of isolated human lipoproteins and rat serum albumin, which were chromatographed in separate experiments. VLDL and LDL were isolated by sequential ultracentrifugation and washed once. Human HDL and RSA were isolated by rate zonal density gradient ultracentrifugation in a swinging bucket rotor, as described by Groot et al.18

In agreement with previously published data, the half-life of labeled HDL-apo A-I (measured between 8 and 28 hours after injection of the labeled HDL) was 10.2 ± 0.7 hours. The serum decay of $^{125}$I-apo A-IV was faster than the serum decay of $^{131}$I-apo A-I. The half-life of $^{125}$I-apo A-IV measured between 8 and 28 hours after injection was 8.5 ± 0.5 hours. In vivo "screening" of the labeled HDL had no effect on the serum decay curves. Most experiments were performed in chow-fed rats but, as shown in Figure 4 B, no changes in the serum decay of radioactive apo A-IV or apo A-I were induced by fasting the animals for 20 hours.

The tissue sites of degradation of HDL-apo A-IV were determined in leupeptin-treated rats according to the methodology described by Van't Hoff et al.13 Leupeptin is a tripeptide that inhibits the lysosomal degradation of proteins, a process that occurs very fast in the absence of the inhibitor. Radioactivity derived from intravenously injected radioactive proteins therefore accumulates in the lysosomes of tissues of leupeptin-treated animals and actively catabolizes these proteins. Figure 5 shows that leupeptin treatment has no effect on the serum decay of the radioactive HDL apolipoproteins measured over a period of 4 hours after injection of the labeled HDL. Furthermore, analyses of sera from leupeptin-treated and saline-treated rats revealed that all radioactivity remained associated with HDL (as shown in Figure 2 for saline-treated rats). Also, leupeptin treatment did not change the mass distribution of apo A-IV between HDL and the "free" apo A-IV fraction. The results of these and previous studies indicate that leupeptin treatment does not influence the turnover of HDL-apo A-IV during the 4-hour experiments.
peptin-treated rats is called the "leupeptin-dependent accumulation" of radioactive apo A-IV or apo A-I. Only the liver and the kidneys showed a significant leupeptin-dependent accumulation of radioactivity (Figures 6 and 7), indicating that these tissues are sites of degradation of HDL apo A-IV and HDL apo A-I. The liver seems to be relatively more important in the uptake and degradation of apo A-IV than in the catabolism of apo A-I. Comparable results were obtained when the double-labeled HDL was screened before the experiments.

Evidence for the intracellular renal localization of apo A-IV was also obtained from immunocytochemical studies by using the horseradish technique. Figure 8 A shows that apo A-IV is located in the cells of the proximal tubuli of leupeptin-treated rats. No intracellular stain can be detected in the cells of the glomeruli. The sections treated with nonimmune rabbit IgG exhibited no staining under conditions identical to those used for visualization and photography of sections treated with antirat apo A-IV (see Figure 8 C). Kidneys from leupeptin-treated rats have an increased intensity of brightly staining granules in the epithelial cells of proximal tubuli (probably reflecting apolipoprotein accumulated in the lysosomes) when compared with kidneys from saline-treated control rats (Figure 8 B). The kidney sections were also analyzed for apo A-I and apo E with the same technique. Essentially, the same localization was observed for all three HDL apolipoproteins. Also, in the case of apo E and apo A-I, the intensity of stain was the highest in leupeptin-treated animals. Minimal staining was present in the lumen of the proximal tubuli.

Discussion

The method of labeling used in the present study opens the possibility of carrying out metabolic studies of HDL specifically labeled in apo A-IV, without disturbing its physiological composition. By using ultracentrifugation, a significant part of the HDL apo A-IV, -apo A-I, and -apo E...
would be "stripped" and recovered in the fraction d > 1.21 g/ml.8,23,24 These changes may result in an altered in vivo metabolic behavior in the rat.25 A study by Funk et al.26 using canine apo E HDL, isolated either by ultracentrifugation or by column chromatography did not show any difference in metabolic behavior.

In the present work, we intended to study the degradation sites of HDL apo A-IV. In rat serum, apo A-IV is present on HDL as well as on a particle called "free" apo A-IV, which is smaller than HDL.8-11 In agreement with our earlier observations,8 it was found that about one-third of the serum apo A-IV was present as "free" apo A-IV in serum obtained from chow-fed rats. It was therefore important to be sure that no label was present in the "free" apo A-IV fraction. Rechromatography of the labeled lipoproteins, incubations of labeled HDL in vitro with rat serum, or intravenous injection showed that 125I-apo A-IV remained associated with the HDL particle. Analysis of the distribution of 125I-apo A-IV in serum during our in vivo experiments revealed that no label was present in the "free" apo A-IV fraction up to 4 hours after injection of labeled HDL-apo A-IV. This indicates that either apo A-IV in HDL is not a precursor of "free" apo A-IV or that the degradation of "free" apo A-IV derived from HDL is very rapid compared to that of HDL-apo A-IV, so that the label cannot be detected in the "free" apo A-IV fraction. The latter possibility seems unlikely since intravenously injected free 125I-apo A-IV showed exactly the same initial rate of disappearance from serum as injected HDL-125I-apo A-IV.

In separate experiments (not shown) it was found that 80% ± 6% (n = 3) and 80% ± 8% (n = 5) of the injected radioactivity of these two preparations respectively, was still present in serum if measured 15 minutes after injection. No label was present in fractions containing "free" apo A-IV after fractionation of the sera by gel filtration. In vitro incubations of the HDL preparation labeled by in vitro incorporation of 125I-apo A-IV and 131I-apo A-I again showed that the labels of HDL are very stable (see Figure 3).

Because 125I-apo A-IV remains associated with the HDL particle, it was possible to study the in vivo metabolism of HDL apo A-IV and to compare it with the metabolic behavior of apo A-I, the major apoprotein of rat HDL. As shown in Figure 4, the serum decay of HDL apo A-IV was faster than the serum decay of HDL-apo A-I. This observation seems to be at variance with data published by Fidge et al.,11 who could not detect any difference in serum turnover of HDL apo A-IV and rat whole HDL, labeled mainly in apo A-I. It must be stressed that in the present study the turnover of HDL apo A-IV and HDL apo A-I was compared directly by a double labeling approach in order to minimize the effects of other experimental conditions. Also, "screening" of the lipoprotein preparations prior to the metabolic studies did not influence the results. An explanation for the faster rate of metabolism of HDL apo A-IV could be related to the presence of small amounts of triglyceride-rich lipoproteins in the serum of nonfasted rats. If there was any exchange of apo A-IV between HDL and triglyceride-rich lipoproteins in the serum of nonfasted rats. If there was any exchange of apo A-IV between HDL and triglyceride-rich lipoproteins, this exchange could increase the serum decay of HDL apo A-IV. However, 125I-apo A-IV was not found to be associated under any conditions with the triglyceride-rich lipoprotein fractions. Moreover, the serum removal rate of
HDL apo A-IV was identical in 20-hour fasted and nonfasted rats. Those studies therefore strongly suggest that under our experimental conditions the removal rate of HDL apo A-IV from rat serum is faster than that of HDL apo A-I, a situation comparable to that in humans.27, 29

The tissue sites of degradation of HDL apo A-IV were determined in the leupeptin-treated rats, as described previously.13 Treatment of rats with leupeptin delays the protein degradation in the lysosomes, causing accumulation of intravenously injected labeled apolipoproteins in those tissues normally involved in the degradation of those proteins. Leupeptin treatment has no effect on serum levels of lipids and apolipoproteins13 nor does it influence the serum decay of HDL apolipoproteins measured over a period of 4 hours (Figure 5).

Recent studies on the degradation sites of apo A-I, either using leupeptin-treated rats13 or the 125I-tyramine cellulose label,14 showed that the rat kidneys play an important role in the degradation of HDL apo A-I. Also, in dogs26 and in humans30, 31 the kidneys are involved in the degradation of plasma HDL. Results of the present study do confirm the role of the rat kidneys in the degradation of HDL apolipoproteins, and extend this role to apo A-IV. The mechanism for the uptake of HDL apolipoproteins by the kidneys is not yet completely understood. Studies on serum HDL cholesteryl ester metabolism have shown that very little is taken up by the kidneys in vivo,5-7 leading to the conclusion that at least part of the HDL apo A-I may be cleared from the circulation separately from the HDL cholesteryl esters.

It was proposed by Glass et al.14 that a small fraction of “free” apo A-I is rapidly filtered by the glomeruli, followed by endocytosis and degradation of the filtered apo A-I in the epithelial cells of the proximal tubuli. The results of studies on the renal catabolism of HDL in the isolated perfused rat kidney28 and in micropertused rabbit proximal straight nephron segments23 are consistent with this hypothesis. The immunochemical localization studies described in this paper show that apo A-IV, as well as apo A-I and apo E (not shown), are present in granules within the cells of the proximal tubuli. Almost no staining was observed in the lumen or on the brushborder membrane of the tubular cells or in the glomeruli. Leupeptin injection resulted in a specific increase of granular staining inside the cells of the tubuli. All these findings support the idea that the degradation of apo A-IV in the kidneys occurs in the lysosomes of the epithelial cells of the proximal tubuli, a conclusion reached earlier for apo A-I.14

The results of the present study also suggest an important role for the liver in the uptake and degradation of HDL apo A-IV. This could indicate a role of HDL apo A-IV in “reverse cholesterol transport,” as suggested by Delamatre et al.10 We showed recently that part of the HDL apo A-IV is present on particles that do not contain apo A-I. These apo A-IV particles could have a relatively rapid turnover and be specifically degraded in the liver. If this hypothesis is true, it would also explain the relatively rapid serum removal rate of apo A-IV, compared with apo A-I (see Figure 4). Detailed studies are needed to clarify the metabolism of the different apo A-IV-containing subfractions and their relationship with cholesterol metabolism.

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