

Cholesterol esters selectively delivered *in vivo* by high-density-lipoprotein subclass LpA-I to rat liver are processed faster into bile acids than are LpA-I/A-II-derived cholesterol esters

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High-density lipoprotein (HDL) subclass LpA-I has been reported to promote cholesterol efflux from mouse adipose cells *in vitro*, whereas subclass LpA-I/A-II has no effect. To investigate whether the apolipoprotein composition of HDL plays a role in the selective delivery of cholesterol esters to the liver *in vivo*, we labelled HDL in its cholesterol ester moiety and separated [³H]cholesterol oleate-labelled HDL into subclasses LpA-I and LpA-I/A-II by immuno-affinity chromatography. Serum decay and liver association of LpA-I and LpA-I/A-II were compared for the apoprotein and cholesterol ester moieties. Both LpA-I and LpA-I/A-II selectively delivered cholesterol esters to the liver with similar kinetics. The kinetics of biliary secretion of

processed cholesterol esters, initially associated with LpA-I or LpA-I/A-II, were studied in rats equipped with permanent catheters in bile, duodenum and heart. For both LpA-I and LpA-I/A-II, liver association was coupled to bile acid synthesis, with an increase in secretion rate during the night. During the first night period, the biliary secretion of LpA-I-derived radioactivity was significantly greater than for LpA-I/A-II. The data indicate that with both LpA-I and LpA-I/A-II selective delivery of cholesterol esters from HDL to the liver occurs, but that cholesterol esters delivered by LpA-I are more efficiently coupled to bile acid synthesis.

INTRODUCTION

Plasma levels of high-density-lipoprotein (HDL) cholesterol are inversely correlated with the incidence of atherosclerosis [1,2]. It has been generally accepted that HDL exerts its protective effect by removing cholesterol from peripheral cells and delivering it to the liver, a process called reverse cholesterol transport [3].

The exact mechanism by which HDL can 'extract' cholesterol from peripheral tissues is still in dispute. It has been proposed that the efflux of cholesterol from cholesterol-loaded cells depends on the desorption of cholesterol from the plasma membrane to cholesterol acceptors [4–6]. An alternative mechanism considers the binding of HDL to be necessary for the translocation of intracellular cholesterol to the plasma membrane and eventually to HDL [7–9]. High-affinity binding sites for HDL have been identified on a variety of cell types, including liver cells [10–14]. The major HDL apolipoproteins apoA-I and apoA-II have both been reported to bind to cells or plasma membranes [15–17].

Within the HDL population, subclasses can be distinguished with different apolipoprotein compositions [18–20]. One subclass contains both apoA-I and apoA-II (LpA-I/A-II particles), whereas apoA-II is absent from the other (LpA-I particles). Levels of LpA-I and LpA-I/A-II have been shown to be affected differently by drug treatment [21]. Clinical studies showed that low HDL levels in coronary artery disease were linked to low LpA-I levels, whereas LpA-I/A-II levels were not different [22–24]. The levels of LpA-I and LpA-I/LpA-II have also been reported to correlate with gender. Females, which are in general more resistant to coronary artery disease, have higher levels of LpA-I [25].

Studies *in vitro* with cholesterol-loaded mouse adipocytes showed that LpA-I was able to induce cholesterol efflux from the

cell, whereas addition of LpA-I/A-II had no effect [20]. However, with other cell types, it has been reported that LpA-I and LpA-I/A-II are nearly equally efficient in promoting cholesterol efflux [26]. *In vivo* in man, it has been demonstrated that apoA-I on LpA-I has a shorter residence time than apoA-I on LpA-I/A-II, suggesting different catabolic pathways [27]. With HepG2 cells it has been reported that LpA-I and LpA-I/A-II differ in apoprotein binding and uptake and in selective delivery of cholesterol esters, though differences were found to be very small [28].

In a previous study *in vivo* we showed that HDL can serve as a cholesterol acceptor [29]. Subsequent delivery to liver parenchymal cells and secretion of radioactivity in the bile were impaired when low levels of HDL were present, thus supporting the role of HDL in reverse cholesterol transport. Selective delivery of HDL cholesterol esters to the liver is specific for parenchymal cells and is coupled to the formation of bile acids [30].

To investigate whether the apolipoprotein composition of HDL is important for the delivery of cholesterol esters to the liver and for the kinetics of bile acid formation, in the present study we compared the fate of cholesterol esters of LpA-I and LpA-I/A-II in the rat.

MATERIALS AND METHODS

Isolation and labelling of HDL

Human HDL was isolated from the blood of healthy volunteers by differential ultracentrifugation as described by Redgrave et al. [31]. HDL ($1.063 < d < 1.21$) was dialysed against 8 mM phosphate-buffered saline/1 mM EDTA, pH 7.4 (PBS/EDTA), and labelled with [³H]cholesterol oleate (Amersham Corp., Arlington Heights, IL, U.S.A.) by exchange from donor particles

as reported previously [30]. Donor ^3H -labelled particles were formed by sonication of egg-yolk phosphatidylcholine (Fluka, Buchs, Switzerland), cholesterol oleate (BDH Chemicals, Poole, Dorset, U.K.) and 250 μCi of [$1\alpha,2\alpha(n)$ - ^3H]cholesteryl oleate (Amersham). The phosphatidylcholine/cholesterol oleate mass ratio was 60:1. Sonication was carried out with a MSE Soniprep 150 for 40 min (amplitude 12 μm) at 52 °C under a constant stream of N_2 in 0.1 M KCl/10 mM Tris/1 mM EDTA/0.025 % NaN_3 buffer, pH 8.0. Donor particles of density approx. 1.03 g/ml were isolated by density-gradient ultracentrifugation. Phospholipid content of the particles was measured by an enzymic colorimetric assay (Phospholipids Kit, from Boehringer, Mannheim, Germany).

HDL was labelled by incubating HDL with donor particles (mass ratio HDL protein:particle phospholipid = 8:1) in the presence of human lipoprotein-deficient serum for 5 h at 37 °C in a shaking water bath under N_2 . Radiolabelled HDL was re-isolated by density-gradient ultracentrifugation, dialysed and passed through a heparin–Sephadex affinity column to remove apo E [32]. The apo E-free fraction was checked for the presence of apo E and albumin by SDS/PAGE with 5–22.5 % -acrylamide gels, followed by Coomassie Blue staining. Almost all of the radioactivity in HDL could be recovered in the cholesterol ester moieties as determined by Bligh and Dyer extraction [33] and t.l.c.; 2.2 ± 0.1 % of the radioactivity was present as unesterified cholesterol.

Isolation of HDL subclasses

The immunosorbents with monoclonal antibodies directed against apo A-I (A05, A17, A30) and apo A-II (G03, G05, G11) [34,35] were prepared by coupling the antibodies to CNBr–Sephadex 4B at a concentration of 8 mg of ligand/ml in accordance with the procedure of the manufacturer (Pharmacia Fine Chemicals, Uppsala, Sweden).

For isolation of the particles, radiolabelled apo E-free HDL was applied to the anti-apo A-II immunosorbent at a flow rate of 10 ml/h, equilibrated with 0.01 M Tris/0.15 M NaCl/0.01 M EDTA. The column was washed with the same buffer containing 0.5 M NaCl at a flow rate of 60 ml/h to eliminate non-specifically bound proteins. The retained fraction (LpA-I/A-II particles) was then eluted with 3 M NaSCN at a flow rate of 60 ml/h and immediately filtered through a column packed with Sephadex G-25 (coarse grade) to remove most of the thiocyanate from the lipoprotein particles.

The unbound lipoproteins free of apoA-II were then chromatographed on the anti-apoA-I column as described above for the anti-apoA-II column. The fraction retained by the anti-A-I column corresponded to the LpA-I particles. LpA-I and LpA-I/A-II particles were finally dialysed against PBS/1 mM EDTA and checked for the presence or absence of apolipoproteins by electrophoresis in SDS gels. The chemical composition of the particles was determined by using commercial enzyme kits (Boehringer) for cholesterol, triacylglycerols and phospholipids. Analysis of apolipoprotein composition was carried out by an e.l.i.s.a. with monoclonal antibodies. Radioiodination of ^3H -LpA-I and ^3H -cholesteryl ester-labelled LpA-I/A-II was carried out by a modification [36] of the ICl method described by McFarlane [37].

Serum decay and liver association

Male Wistar rats (12 weeks old) were anaesthetized by intraperitoneal injection of Nembutal (80 mg/kg body wt.). The body temperature was maintained at 36.5–37 °C by an i.r.

heating lamp, monitored as the rectal temperature. After the abdomen was opened, radiolabelled LpA-I and LpA-I/A-II were injected into the vena penis. Single-labelled (^3H)cholesterol oleate) LpA-I or LpA-II/A-II was injected in order to determine the serum decay and liver association of the cholesterol ester moiety, whereas double-labelled ($^{125}\text{I}/^3\text{H}$) particles were injected to study the clearance of the apoprotein moiety. At the indicated time points, blood sampling and liver lobule excision were performed as described previously [38]. Liver and serum samples of experiments with ^3H cholesterol ester-labelled LpA-I or ^3H cholesterol ester-labelled LpA-I/A-II were combusted in a Packard sample oxidizer and counted for radioactivity in a Packard liquid-scintillation counter. Samples from $^{125}\text{I}/^3\text{H}$ -labelled particles were counted in a Packard γ -radiation counter. To calculate liver-associated radioactivity, corrections were made for the contribution of serum to the total liver-associated radioactivity.

Bile sampling

Bile was collected from unrestrained 3-month-old male Wistar rats, as reported previously [39]. Rats received tap water and standard chow *ad libitum*. Rats were equipped with permanent catheters in the bile duct, the duodenum and the heart. Bile-duct and duodenum catheters were connected immediately after surgery in order to maintain an intact enterohepatic circulation. Rats were allowed to recover from surgery for 1 weeks. Then 100–150 μg of LpA-I or LpA-I/A-II was introduced via the heart catheter, and the bile-duct catheter was connected to a fraction collector. Bile samples were collected hourly. A 500 μl portion of bile was de-colourized by adding 100 μl of 75 % H_2O_2 solution. Hionic fluor scintillation fluid (Packard) was added, and the samples were counted for radioactivity in a Packard liquid-scintillation analyser.

Protein determination

Protein was determined as described by Lowry et al. [40], with BSA (Sigma, St. Louis, MO, U.S.A.) as standard.

RESULTS

The chemical compositions of ^3H cholesterol ester-labelled LpA-I and ^3H cholesterol ester-labelled LpA-I/A-II particles are given in Table 1. Almost all of the protein moiety of LpA-I was apoA-I (99.1 %), whereas for LpA-I/A-II the apoA-I:apoA-II:apoC-III proportions were 53.9:43.0:0.2. The chemical composition (protein, cholesterol, triacylglycerols and phospholipids) was similar for LpA-I and LpA-I/A-II.

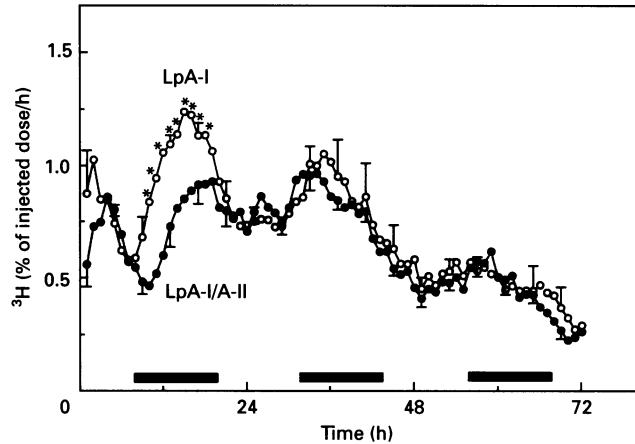
In order to determine whether both LpA-I and LpA-I/A-II could selectively deliver cholesterol esters to the liver, the apoprotein moiety of the particles was labelled by iodination. Figure 1 depicts the serum decay and liver association of LpA-I (1a) and LpA-I/A-II (1b). It appears that the initial serum decay kinetics of LpA-I and LpA-I/A-II are similar, and also liver uptake is identical. At 60 min after injection of LpA-I the liver association of the iodinated apoprotein was 8.2 ± 1.0 % of the injected dose, whereas for the ^3H cholesterol ester moiety 22.9 ± 1.3 % was recovered in the liver. For LpA-I/A-II these values were 8.9 ± 0.5 % and 25.3 ± 1.3 % respectively. The data for LpA-I and LpA-I/A-II were not significantly different. Selective delivery of cholesteryl esters to the liver thus occurred with both LpA-I and LpA-I/A-II, indicating that the process of selective delivery is independent of this variation in apolipoprotein composition.

Table 1 Chemical composition of LpA-I and LpA-I/A-II

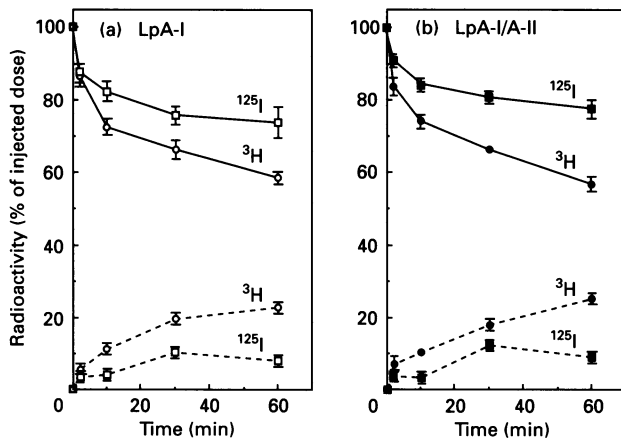
[^3H]Cholesterol oleate-labelled HDL was separated into LpA-I and LpA-I/A-II subclasses by affinity immunochromatography. The chemical composition of the particles from two isolations was determined. ND, not detectable.

	Mass (%)			
	Protein	Cholesterol	Triacylglycerols	Phospholipids
LpA-I	57.2	15.5	ND	27.3
	54.2	16.4	2	27.2
LpA-I/A-II	56.3	14.6	ND	29.1
	57.4	15.1	1.4	26.1

	Apolipoprotein composition (mol%)		
	A-I	A-II	C-III
LpA-I	99.1	ND	0.9
	99.1	ND	0.9
LpA-I/A-II	42.8	57.0	0.2
	64.9	34.9	0.2

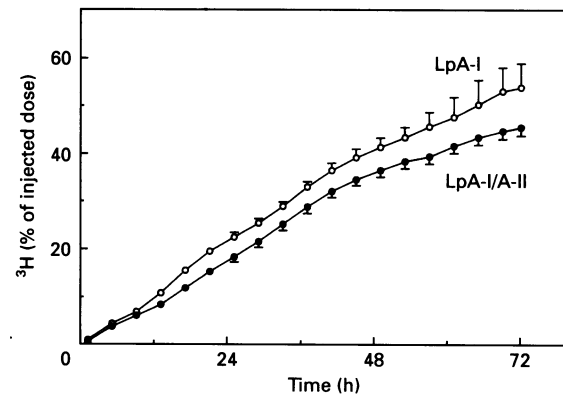
**Figure 2** Biliary secretion of ^3H radioactivity after injection of [^3H]cholesterol ester-labelled LpA-I or [^3H]cholesterol ester-labelled LpA-I/A-II

Bile was collected for 72 h in 1 h time intervals after injection of 100–150 μg (100 000–150 000 d.p.m.) of LpA-I (\circ) or LpA-I/A-II (\bullet) in unrestrained cannulated rats. Values are expressed as percentages of injected dose/h and represent means of six experiments \pm S.E.M. Black horizontal bars indicate the dark periods. Points marked with an asterisk are significantly different ($P < 0.05$).

**Figure 1** Serum decay and liver association of LpA-I and LpA-I/A-II in the rat

Samples (100–150 μg ; 100 000–150 000 d.p.m.) of [^3H]cholesterol ester-labelled LpA-I (\circ , \square) or [^3H]cholesterol ester-labelled LpA-I/A-II (\bullet , \blacksquare) were injected into the vena penis of anaesthetized rats. At the indicated time points, serum was withdrawn from the vena cava inferior and a liver lobule was tied off, weighed, combusted in a Hewlett–Packard sample oxidizer 306 and counted for radioactivity. Serum decay (—) and liver association (----) were calculated. A correction was made for the contribution of serum to the total liver-associated radioactivity [38]. The data for the cholesterol ester moiety are compared with data for the ^{125}I -apolipoprotein moiety of LpA-I and LpA-I/A-II respectively (\square , \blacksquare). Data are expressed as percentage of injected dose \pm S.E.M. ($n = 4$).

To investigate the kinetics of biliary secretion of initially LpA-I- and LpA-I/A-II-associated cholesterol esters, rats were equipped with permanent catheters in the bile duct, duodenum and heart. Since only a small amount of LpA-I and LpA-I/A-II (100–150 μg) was injected, no mass effects were induced by administration of the particles. The biliary secretion of radioactivity, after injection of radiolabelled LpA-I and LpA-I/A-II,

**Figure 3** Biliary secretion of ^3H radioactivity after injection of [^3H]cholesterol ester-labelled LpA-I or LpA-I/A-II

Bile was collected for 72 h in 1 h time intervals after injection of 100–150 μg (100 000–150 000 d.p.m.) of LpA-I (\circ) or LpA-I/A-II (\bullet) in unrestrained cannulated rats. Data from Figure 2 are expressed as percentage of injected dose \pm S.E.M. ($n = 6$). For clarity data points are shown every 4 h.

expressed as percentage of injected dose per hour is shown in Figure 2. For LpA-I a clear day/night rhythm can be observed. The marked increase in secretion of radioactivity in the bile during the first dark period for LpA-I is less evident for LpA-I/A-II. For LpA-I/A-II the increase is delayed and reaches a lower level than for the LpA-I particles. In the first dark period (8–20 h after injection) the secretion of radioactivity derived from LpA-I is thus significantly higher than for LpA-I/A-II (Student t test, $P < 0.05$). For the reason that no mass changes occur in bile secretion, it appears that the specific radioactivity is higher for LpA-I than for LpA-I/A-II. The higher biliary secretion rate in the first dark period for LpA-I leads to a higher biliary secretion when expressed cumulatively (Figure 3). For

both particles, radioactivity was secreted mainly as bile acids ($89 \pm 2\%$ and $81 \pm 6\%$ for LpA-I and LpA-I/A-II respectively).

DISCUSSION

Human HDL is a heterogeneous population of particles that differ in size, density, apoprotein and lipid composition. In this study we isolated HDL by preparative ultracentrifugation, labelled HDL with [^3H]cholesterol oleate and re-isolated HDL by a second ultracentrifugation step. To avoid interaction of HDL with apoE-binding sites in the liver, the radiolabelled HDL was made apoE-free. In the concept of reverse cholesterol transport, the liver is of major importance as a cholesterol-accepting and degrading tissue. We have shown previously [30] that HDL, isolated and labelled as described here, is able to exert selective cholesterol ester delivery specifically to the liver parenchymal cells. We have also shown that in the liver [^3H]cholesterol esters are efficiently metabolized to radiolabelled bile acids had secreted into the bile. In this study we further analysed the particular importance of HDL subclasses LpA-I and LpA-I/A-II for this pathway.

Studies *in vitro* concerning the binding of HDL subclasses LpA-I and LpA-I/A-II and subsequent cholesterol efflux from the cells have indicated that LpA-I has a greater potency to decrease cellular cholesterol levels [41,42]. In the present study we isolated the subclasses LpA-I and LpA-I/A-II from cholesterol-ester-labelled HDL by immunoaffinity chromatography and studied the behaviour *in vivo* of [^3H]cholesterol esters initially associated with LpA-I and LpA-I/A-II.

It can be concluded that cholesterol esters initially associated with LpA-I and LpA-I/A-II are both selectively cleared from the circulation as compared with the iodinated apolipoprotein moiety. At 60 min after injection, the liver uptake of the [^3H]cholesterol esters is 2.8-fold greater than that of the [^{125}I]apoprotein moiety. Both subclasses deliver their cholesterol esters to the liver to the same percentage, which indicates that cholesterol esters, initially associated with LpA-I or LpA-I/A-II particles, reach the liver with similar kinetics. Of course we cannot exclude that redistribution of apolipoproteins *in vivo* occurs. However, experiments *in vivo* with radiolabelled apolipoproteins in man suggested that a rapid exchange of apolipoproteins is limited and that at least part of the apolipoproteins exchanges at a slower rate than the catabolic rate [27]. Moreover, as mentioned below, the difference in kinetics of biliary secretion support the possibility that the liver can discriminate between the two different classes of injected particles. Experiments *in vitro* with pig liver and adrenal membranes show a greater binding of LpA-I than of LpA-I/A-II [43]. With HepG₂ cells the greater binding of LpA-I was coupled to a slightly increased uptake of cholesteryl linoleyl ethers [28]. The specific binding of LpA-I and LpA-I/A-II by HepG₂ cells, however, is much lower than the binding of HDL to isolated rat parenchymal cells [13]. Furthermore, bile acid biosynthesis by HepG₂ cells is different from that in rat hepatocytes [44] and does not exhibit clear day/night rhythms.

By using bile-catheterized rats we were able to study the kinetics of biliary secretion of radioactivity derived from [^3H]cholesterol-ester-labelled LpA-I and LpA-I/A-II. A marked difference in biliary secretion between LpA-I and LpA-I/A-II was noticed in the first dark period (8–20 h after injection). The day/night rhythm of biliary secretion was much more evident for LpA-I than for LpA-I/A-II particles. The higher secretion rate in the first night leads to a higher percentage of radioactivity secreted in the bile for LpA-I when expressed as cumulative

percentage of injected dose. For both ligands, radioactivity was recovered mainly as bile acids, indicating that the metabolic pathways for the particles were similar.

Since we did not detect a significant difference between serum decay and liver association of [^3H]cholesterol-ester-labelled LpA-I and [^3H]cholesterol-ester-labelled LpA-I/A-II, the greater biliary secretion of LpA-I-derived radioactivity must be exerted after the initial uptake of cholesterol esters. It may be expected that specifically the binding of LpA-I to the liver parenchymal cell *in vivo* stimulates an efficient intracellular cholesterol transport to bile-acid-synthesizing enzymes. Although the presence of apoA-II on the LpA-I/A-II does not have implications for the delivery of cholesterol esters to the liver, it may hamper the further processing of HDL cholesterol esters to bile acids. Further work on the mechanism of selective delivery and its coupling to bile acid formation will, however, be necessary in order to analyse the precise mechanism of the effects of apoA-I versus apoA-I/A-II on this process.

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