Intravenous apoA-I/lecithin discs increase pre-β-HDL concentration in tissue fluid and stimulate reverse cholesterol transport in humans

M. N. Nanjee,* C. J. Cooke,* R. Garvin,* F. Semeria,* G. Lewis,* W. L. Olszewski,*,† and N. E. Miller1,*

Department of Cardiovascular Biochemistry,* St. Bartholomew’s and Royal London School of Medicine and Dentistry, Charterhouse Square, London EC1M 6BQ, UK; and Department of Surgical Research and Transplantology,† Medical Research Center, Polish Academy of Sciences, Warsaw, Poland

Abstract The extent to which plasma HDL concentration regulates reverse cholesterol transport (RCT) is not known. The principal acceptors of unesterified cholesterol (UC) in humans are small pre-β-HDL, which we have shown in plasma during intravenous infusion of apoA-I/lecithin (apoA-I/PL) discs in humans. We have now examined the effects on tissue fluid HDL and RCT. ApoA-I/PC or proapoA-I/PC discs were infused into 16 healthy males. Eleven had been given intravenous radiocholesterol to label tissue pools; in 12 prenodal leg lymph was collected throughout; and in 8 all feces were collected. The rise in small pre-β-HDL in plasma associated with increases in 1) pre-β-HDL concentration in lymph (all subjects), 2) the size of other lymph HDL (four of four subjects), 3) the cholesterol content of lymph lipoproteins relative to plasma lipoproteins (P < 0.01, n = 4), 4) cholesterol-specific radioactivity in lymph (five of nine subjects), 5) plasma lathosterol (P < 0.004, n = 4), 6) plasma cholesterol esterification rate (P < 0.001, n = 4), and 7) fecal bile acid excretion (P < 0.001, n = 8). These results support the hypothesis that small pre-β-HDL generated in plasma readily cross endothelium into tissue fluid, and thereby promote efflux of UC from peripheral cells. After delivery to the liver, peripheral cholesterol appears to be utilized more for bile acid synthesis than for biliary cholesterol secretion in humans.—Nanjee, M. N., C. J. Cooke, R. Garvin, F. Semeria, G. Lewis, W. L. Olszewski, and N. E. Miller. Intravenous apoA-I/lecithin discs increase pre-β-HDL concentration in tissue fluid and stimulate reverse cholesterol transport in humans. J. Lipid Res. 2001. 42: 1586–1593.

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Studies of animals have shown that HDL have antiatherogenic activity, but the mechanism is not known. One function of HDL is the transport of cholesterol from peripheral cells to the liver (reverse cholesterol transport, RCT) (1). When fibroblasts are exposed to plasma, the primary acceptors of cell-derived unesterified cholesterol (UC) are small lipid-poor apolipoprotein A-I (apoA-I)-containing pre-β-HDL (2). Cell-derived UC is then esterified by LCAT, and the resultant cholesteryl esters (CE) accumulate in spheroidal a-HDL (2). Transfer of CE from a-HDL to hepatocytes is mediated by scavenger receptor class B type I (SR-BI) receptors (3). In some species (including humans) CE are also transferred from a-HDL to apoB-containing lipoproteins via CETP (1). After uptake by hepatocytes, HDL cholesterol appears to be utilized for bile acid synthesis (4).

The extent to which plasma HDL regulate RCT is not known. The rate of efflux of UC from cultured cells is influenced by HDL concentration, and this effect has been attributed to the pre-β-HDL (5). However, studies of animals have not provided consistent evidence that RCT is influenced by plasma HDL concentration in vivo. Although the clearance of LDL cholesterol from muscle was reduced in apoA-I knockout mice (6), it was not increased in human apoA-I transgenic mice (7). Furthermore, apoA-I knockout mice showed no reduction of cholesterol synthesis or LDL receptor activity in peripheral tissues (8).

We have shown that intravenous infusion of apoA-I/phosphatidylcholine (PC) discs rapidly increased plasma pre-β apoA-I and HDL cholesterol concentrations in humans (9). As the discs had no effect on the UC content of erythrocytes in vitro, our results suggested that the infusion had stimulated RCT. However, the possibility that the rise in HDL cholesterol reflected uptake of UC from the liver only, or diminished transport of CE from HDL by

Abbreviations: apo, apolipoprotein; CE, cholesteryl ester; HP-SEC, high-performance size exclusion chromatography; PC, phosphatidylcholine; PL, phospholipids; RCT, reverse cholesterol transport; SR-BI, scavenger receptor class BI; UC, unesterified cholesterol.

1 To whom correspondence should be addressed.

E-mail: n.e.miller@mds.qmw.ac.uk
CETP, was not excluded. Nor was it shown that cholesterol elimination via the liver was enhanced.

In this study we tested the hypothesis that the pre-β-HDL that are generated in plasma during infusion of apoA-I/PC or proapoA-I/PC discs cross the endothelium, and thereby enhance cholesterol flux through the three anatomical components of RCT: transfer of UC from cells to HDL in tissue fluid; esterification of HDL UC in plasma by LCAT; and conversion of HDL CE to bile acids in liver.

MATERIALS AND METHODS

Subjects

Sixteen healthy males were studied (Table 1). None was taking a special diet or medication. The study was approved by the local ethics committee, and all subjects gave informed consent.

Clinical procedures

Sixty-eight to 102 days before the study, 11 of the subjects were given an intravenous infusion of 50–100 μCi of [4-14C]cholesterol (Amersham Pharmacia Biotech, Piscataway, NJ) in 0.5 ml of ethanol, dispersed in 500 ml of 0.15 mM NaCl, to label tissue cholesterol (10).

All subjects were studied in a metabolic ward, consuming a eucaloric solid diet [cholesterol, 186 mg/day; fat, 65 g/day; saturated:polyunsaturated:monounsaturated fatty acids (% weight), 21:20:79], from which cholesterol (10).

Subjects Age Weight BMI

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Laboratory procedures

Blood and lymph tubes were placed on ice, and then centrifuged (1,500 g, 15 min, 4°C). Plasma and lymph from the same subject were processed together. All assays were done in duplicate.

Lipids and apolipoproteins. Total cholesterol, UC, CE, TG, and total choline-containing phospholipids (PL) were quantified as previously described (9). Precinorm L® (Roche Diagnostics, Indianapolis, IN) was used as calibrator. Albumin and apoA-I, apoAII, apoB, apoC-III, and apoE were quantified by radioimmunoassays or rocket immunoelectrophoresis (9).

ApoA-I in HDL subclasses. The distribution of apoA-I in pre-β and α migrating particles was determined by crossed immunoelectrophoresis (9). ApoA-I concentrations in the two regions were then calculated by reference to the total apoA-I concentration in the sample.

Size subclasses of apoA-I-containing particles were separated by high performance-size exclusion chromatography (HP-SEC) through Superdex 200 and Superdex 75 gel-permeation col
This separates the particles into three subclasses: a major population of 70–500 kDa, composed of CE-rich α-HDL; a minor population of >500 kDa; and a minor population of small particles (40–60 kDa) that have pre-β electrophoretic mobility, and appear to be a mixture of lipid-free apoA-I and lipid-poor HDL (i.e., pre-β₂-HDL) (15).

**Cholesterol in HDL and size subclasses of HDL.** Plasma HDL lipids were measured after precipitation of apoB-containing lipoproteins with 8% polyethylene glycol (PEG) 8000 (9). In some subjects cholesterol was assayed in size subclasses of lipoproteins separated through Superose 6 (9).

**Cholesterol-specific radioactivity.** Aliquots of whole plasma, apoB-free plasma (PEG supernatant), or whole lymph were mixed with 10 volumes of OptiPhase Trisafe scintillant (Perkin-Elmer Life Sciences, Boston, MA) and counted at >95% efficiency in a liquid scintillation spectrometer to <1% error. Cholesterol mass was assayed as described.

**Plasma cholesterol esterification rate.** Cholesterol esterification rate in plasma was assayed by quantifying the decrease in UC concentration enzymatically during incubation in vitro at 37°C for 3 h (16).

**Plasma lathosterol.** Plasma lathosterol concentration, an index of whole body cholesterol synthesis, was quantified by gas-liquid chromatography, as described by Kempen et al. (17), using 5α-cholestanol (Sigma-Aldrich) as internal standard.

**Fecal steroid excretion.** Fecal neutral and acidic steroids were quantified as their trimethylsilyl ethers by gas-liquid chromatography, as described by Czubayko et al. (14). Internal standards were 5α-cholestanol and hyodeoxycholic acid for neutral and acidic steroids, respectively. Daily excretion rates were calculated by expressing the results relative to stigmastanol (14). Cholic acid excretion was calculated as the sum of cholic, deoxycholic, and isodeoxycholic acids. Chenodeoxycholic acid excretion was calculated as the sum of chenodeoxycholic, iso胆酸enodeoxycholic, lithocholic, isolithocholic, and ketolithocholic acids.

**Statistical analyses**

Changes in concentrations were analyzed by repeated measures analysis of variance and by Fisher’s protected least-squares difference. \( P < 0.05 \) was regarded as significant. Analysis of the data was complicated by the fact that protein and lipoprotein concentrations in lymph exhibit circadian variations (18, 19), owing to the changes in posture and physical activity during the day-night cycle, and their effects on the transport of water and solutes across endothelium (19). We used two approaches to overcome or adjust for this phenomenon: 1) limiting comparisons of concentrations in lymph to those at the same 24-h clock time; and 2) expressing concentrations in lymph relative to those of albumin.

**RESULTS**

Changes produced by the two preparations of discs were qualitatively similar, although those produced by the apoA-I/PC discs were usually the greater.

**Plasma and lymph apolipoproteins and lipids**

Plasma apoA-I and HDL PL increased during the infusion (Fig. 1A and B). Thereafter, HDL PL decreased with a half-life about half that of apoA-I. Non-HDL PL was unchanged. The apoA-I/albumin and PL/albumin ratios in lymph increased more slowly, reaching peak values after about 18 h (Fig. 1D and E).

The concentrations of apoA-II, apoB, and apoE in plasma and the apoA-II/albumin, apoB/albumin, and apoE/albumin ratios in lymph were unaffected by the infusion (not shown). The corresponding values for apoC-III increased slightly (not shown).

Plasma HDL UC increased during the infusion, and then decreased as HDL CE rose (Fig. 1C). Plasma non-HDL CE increased by a smaller amount (not shown). The UC/albumin and CE/albumin ratios in lymph rose more slowly, being progressively more evident at the maximum values in the 24 h cycle (Fig. 1F).

In four subjects cholesterol was quantified in size subfractions after Superose 6 HP-SEC (Fig. 2). HDL cholesterol increased in both matrices, accompanied by a shift toward larger particles. The shift occurred more slowly and to a greater degree in lymph than in plasma.

Evidence of enrichment of lymph lipoproteins with cholesterol was sought by monitoring the changes in the
cholesterol/apoA-I and cholesterol/(apoA-I plus apoB) ratios. Both ratios decreased in plasma, an expected consequence of infusion of cholesterol-free apoA-I-containing particles (Fig. 3). They also decreased in lymph, presumably secondary to the changes in plasma, but the reductions here were much smaller than those in plasma, providing evidence of increased efflux of cholesterol from cells into tissue fluid.

We have reported that the concentration of apoA-I in small pre-β-HDL increased during a 4-h infusion of apoA-I/PC discs, and then decreased to baseline values during the following 4–6 h (9). The longer duration of the present study revealed that the time course of plasma pre-β apoA-I was often biphasic, the concentration rising again 24 h or more later (Fig. 4). Pre-β apoA-I also increased in lymph, but here it took 18–24 h to reach peak value, and it then remained elevated for 60 h or more (Fig. 4). Superdex HP-SEC showed that the concentration of small apoA-I-containing particles in lymph increased postinfusion. This was again slower to develop, and was associated with a greater shift toward larger particles than in plasma (Fig. 5).
0.0001), third (+144%, $P = 0.03$), and seventh (+192%, $P = 0.007$) 24-h periods postinfusion. Although neutral steroid excretion also rose, this was not significant ($P = 0.08$).

**DISCUSSION**

We have previously shown that infusion of apoA-I/PC discs in humans rapidly increased the plasma concentrations of apoA-I in small pre-β-HDL and HDL UC, followed by increases in apoA-I in large α-HDL and HDL CE (9). Studies in vitro suggested that the increase in HDL cholesterol could not be attributed solely to transfer of UC from other lipoproteins or erythrocytes. Thus, it appeared that the discs themselves and/or the small pre-β-HDL generated during the infusion had mobilized UC from one or more fixed tissues. However, it could not be concluded that RCT from peripheral cells had been stimulated.

The objectives of the present study were 3-fold: to determine the longer term effects of disc infusion on plasma HDL; to characterize the changes in tissue fluid lipoproteins; and to test the hypothesis that these are associated with increases in the extravascular, intravascular, and intrahepatic components of RCT. Our findings confirmed the acute effects on plasma HDL. The decline in plasma total apoA-I postinfusion was consistent with the half-life of endogenous apoA-I in humans (20), suggesting that the exogenous apoA-I was metabolized physiologically. Our present results showed that the rise in plasma HDL cholesterol persists for more than 72 h, and that the transient rise in plasma pre-β-HDL during the infusion is followed by a second rise after about 24 h. In accordance with our earlier evidence that plasma pre-β-HDL concentration influences that in tissue fluid (21), pre-β-HDL also increased in lymph, but more slowly and for longer. The probable explanation for the differing time courses in plasma and lymph is that the intravascular production of pre-β-HDL was acutely stimulated during the infusion. The subsequent decline in their concentration in plasma resulted from two processes: conversion to spheroidal α-HDL by LCAT; and transfer across capillary endothelium. The latter process increased their concentration in lymph. The late postinfusion rise in plasma pre-β-HDL reflected the return of these itinerant particles to blood via the thoracic duct.

If the above-described interpretation is correct, the rise in HDL UC during the infusion will have reflected efflux of UC not from peripheral cells, but from tissues that are unusually accessible to plasma HDL, such as liver and spleen. An acute decrease in the UC pools in these organs would stimulate cholesterol synthesis, explaining both the early rise in plasma lathosterol concentration and the acute fall in plasma cholesterol-specific radioactivity.

Mobilization of UC from peripheral cells is likely to have occurred only after sufficient apoA-I-containing particles had crossed endothelium into tissue fluid. Evidence that the rise in lymph pre-β-HDL was associated with cholesterol efflux from peripheral cells was provided by our
cholesterol-specific radioactivity measurements. As observed by Reichl et al. (22), under basal conditions the specific radioactivity of lymph cholesterol exceeded that of plasma cholesterol, reflecting the origin of much of the former from peripheral tissues. Within plasma the specific radioactivity of HDL cholesterol exceeded that of non-HDL cholesterol. Counting rates were too low for lymph HDL cholesterol-specific radioactivity to be determined. After the disc infusion, lymph cholesterol-specific radioactivity increased in some subjects, indicative of an increase in the efflux of cholesterol from tissues. This was most evident in the first lymph samples collected in the morning. As the HDL in these samples will have been exposed to tissues for longer than average, owing to the fact that the flow of lymph is slowest overnight (19), they would be expected to manifest the effects of cholesterol mobilization to the greatest extent. Increases in the UC/albumin and CE/albumin ratios in lymph, although not statistically significant, were also most apparent in the morning.

The re-entry of HDL carrying tissue cholesterol of high specific radioactivity into blood via lymph explains the return of plasma cholesterol-specific radioactivity toward preinfusion values, and sometimes higher, after its initial decrease. The rise in plasma cholesterol esterification rate indicates that the flux of CE through plasma HDL was also increased. As large discoidal HDL are the optimum substrate for plasma LCAT (23), this may have been a direct effect of the infused particles acting as a substrate after acquiring UC. However, the increase in percentage esterification rate persisted for too long for this to be the full explanation, suggesting that it was also due in part to the remodeling of plasma HDL and/or to the re-entry into plasma of lymph pre-β-HDL carrying tissue-derived UC.

Fig. 5. Effects of apoA-I/PC disc infusion on apoA-I in size subfractions of plasma and lymph separated by HP-SEC through Superdex 200 and 75 in series. Representative results from a single subject (no. 16). Results in the top two panels are the absolute concentrations at $t=0$ h. The other panels show the changes at the indicated times relative to the $t=0$ h profile (in the case of plasma) or relative to the profile 24 h before (in the case of lymph, to allow for the circadian variation). In the top two panels CE-rich spheroidal HDL elute in fractions 10–42, and small (40- to 60-kDa) lipid-poor apoA-I-containing particles elute in fractions 43–55; peak elution of albumin occurs in fraction 40. No HP-SEC was performed on lymph samples at $t=4$ or $t=8$ h, as little change had occurred in lymph total apoA-I at these time points.

Fig. 6. Effects of apoA-I/PC disc infusion on plasma lathosterol concentration and lathosterol/cholesterol ratio (A), and the mass and percentage cholesterol esterification rates during incubation of plasma in vitro (B). Means ± SEM from four subjects (nos. 13–16).
The rise in the fecal excretion of cholate and its derivatives can be completely explained by the elimination of the cholate that is a component of the apoA-I/PC discs. The infusates will have contained on average 851 mg of cholate (13), which we have shown is rapidly cleared from plasma (9), presumably by the liver. However, the rise in chenodeoxycholate excretion cannot be explained in this way (24). As this was also unlikely to have been due to the initial rise in intrahepatic cholesterol synthesis (secondary to a decrease in the intrahepatic UC pool), it was presumably in response to the rise in plasma HDL CE, either directly or after transfer of CE to other lipoproteins. In rats HDL CE are utilized for bile acid synthesis in preference to LDL cholesterol (4). Price, Cortese, and Miller (25) showed that lipoprotein CE are converted more rapidly to bile acids than to biliary cholesterol in humans. Eriksson et al. (26) examined the effects of infusion of proapoA-I/PC discs on fecal steroids in four patients with familial hypercholesterolemia. Results obtained for both neutral and acidic steroids 9–12 days after the infusion were significantly greater than those 6–9 days before the infusion, but no results were reported for other days. Thus, our findings provide evidence that each of the three anatomical components of RCT is increased by intravenous infusion of reconstituted HDL. This suggests that native nascent HDL probably have a similar effect.

Previous studies of the effect on RCT of a change in HDL production rate have been carried out in mice. The peripheral tissues of apoA-I knockout mice (8) had values for cholesterol content, cholesterol synthesis, and LDL receptor activity similar to those of normal mice, implying that RCT was unaffected. Stein et al. (7) found that the clearance from muscle of cholesterol injected in cationized LDLs was normal in apoA-I transgenic mice. Although cholesterol clearance from muscle was reduced in apoA-I knockout mice, the effect was modest compared with the reduction in plasma HDL cholesterol (6). These results do not necessarily conflict with our findings. As mice have no CETP activity in plasma, the transfer of CE from HDL to the liver via SR-BI receptors is normally saturated in this species (27). Under such circumstances, variations in apoA-I synthesis might not have a major effect on steady state RCT. However, they might have an effect when, as in humans, CETP provides a second route of exit for CE from plasma HDL.

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REFERENCES

Nanjee et al. ApoA-I/PC discs and reverse cholesterol transport 1593