Stimulation of Fecal Steroid Excretion After Infusion of Recombinant Proapolipoprotein A-I
Potential Reverse Cholesterol Transport in Humans

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Background—Apolipoprotein (apo) A-I is the major protein component of HDL, a cholesterol transport particle that protects against atherosclerosis. Apo A-I is believed to promote reverse cholesterol transport, transferring cholesterol from peripheral cells to the liver for subsequent elimination. To test this hypothesis in humans, we measured fecal steroid excretion before and after the intravenous infusion of human proapo A-I (precursor of apo A-I) liposome complexes.

Methods and Results—Four subjects with heterozygous familial hypercholesterolemia were studied under standardized conditions. The fecal excretion of bile acids and neutral sterols was determined for 9 days before and 9 days after an intravenous infusion of recombinant human proapo A-I (4 g protein) liposome complexes. Plasma apoA-I and HDL cholesterol levels increased transiently (mean peak concentrations were 64% and 35% above baseline, respectively) during the first 24 hours. Mean lipoprotein lipid and apolipoprotein levels were not different during the 2 collecting periods, however. Serum lathosterol, a precursor of cholesterol whose concentration reflects the rate of cholesterol synthesis in vivo, was also unchanged. The fecal excretion of cholesterol (neutral sterols and bile acids) increased in all subjects (mean increase, +39% and +30%, respectively), corresponding to the removal of ~500 mg/d excess cholesterol after infusion. Control infusions with only liposomes in 2 of the patients did not influence lipoprotein pattern or cholesterol excretion.

Conclusions—Infusion of proapoA-I liposomes in humans promotes net cholesterol excretion from the body, implying a stimulation of reverse cholesterol transport. This mechanism may prove useful in the treatment of atherosclerosis.

Key Words: apolipoproteins ■ atherosclerosis ■ cholesterol ■ lipoproteins ■ metabolism

The risk of developing coronary heart disease is directly related to plasma concentrations of total and LDL cholesterol and inversely related to HDL cholesterol levels.1,2 Although the mechanisms for regulation of plasma LDL cholesterol and its role in the process of atherogenesis are relatively well understood,3,4 it is still unclear how HDL may exert its protective actions.5–7 HDL comprises a mixture of particles that differ in size and composition and undergo continuous metabolic changes in the circulation. Nascent HDL is formed in the liver and intestine, where its major apolipoprotein, apoA-I, is secreted as a propeptide (proapoA-I) that is rapidly cleaved to mature apoA-I.1–7 The findings that infusion of HDL particles or apoA-I liposomes, as well as overexpression of the human gene for apoA-I, inhibits the disease process in animal models of atherosclerosis8–13 support the concept of a important role for apoA-I in the prevention of atherogenesis.

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ApoA-I is believed to play a major role in the process of reverse cholesterol transport, ie, the transfer of cholesterol from peripheral tissues to the liver.5–7,14,15 HDL particles, as well as apoA-I liposomes, can remove cholesterol from cultured cells, including cholesteryl ester–loaded macrophage foam cells.15,16 After esterification by the enzyme lecithin-cholesterol acyltransferase, cholesterol can be delivered to steroidogenic tissues, such as the liver, adrenal glands, and gonads. This may occur either via exchange with triglyceride-rich lipoproteins or LDL and subsequent tissue uptake by lipoprotein receptors5–7,17 or directly via specific HDL “docking” sites that recognize apoA-I.18–21 The liver is of critical importance in maintaining reverse cholesterol transport, because it is the only organ from which substantial net excretion of cholesterol can occur, either directly or after conversion to...
If transfer of cholesterol from peripheral tissues to the liver could be stimulated and a net increase in cholesterol excretion achieved, this might have important implications in the treatment of atherosclerosis. To evaluate whether such a stimulation of reverse cholesterol transport could be obtained in humans, we studied the effect of infusion of recombinant human proapoA-I on the fecal excretion of cholesterol and bile acids in hypercholesterolemic subjects.

Methods

Patients
Four male patients (S.G., G.G., L.M., and I.N.) were studied. They were 43, 61, 43, and 42 years old and weighed 87, 85, 97, and 80 kg, respectively. All had phenotypical heterozygous familial hypercholesterolemia, and the first 2 were brothers carrying the FH-Helsinki mutation. Patients S.G. and L.M. had coronary heart disease; all patients were in stable clinical condition on unchanged therapy (β-blockers in S.G., G.G., L.M.) during the study. The study was approved by the Ethics Committee of the Karolinska Institute, and informed consent was obtained from all subjects.

Experimental Procedure
The patients were studied before and after the intravenous infusion of a large amount of cholesterol-free, proapoA-I liposomes. All lipid-lowering drugs were withdrawn 6 weeks before the patients entered the study, which was performed at the metabolic ward on an outpatient basis, with daily visits. The patients were given a standardized, natural-type diet adjusted to keep their weight and cholesterol and fat intake stable. This diet contains 3.5 mg · kg⁻¹ · day⁻¹ of cholesterol; fat content is ~35%. Nine days before infusion, fecal collections were started in 3-day portions, and the elimination of cholesterol from the body was determined. Chromic oxide was given during the whole study period to normalize for variations in fecal flow. At the time of infusion, fecal collections were stopped, the patients were given a capsule of red stain, and complete fecal collection for another 9 to 12 days was restarted when the stain appeared in the feces.

ProapoA-I Liposome Infusion
During the infusion, 200 mL of recombinant human proapoA-I soybean phosphatidylcholine liposomes containing 4 g of the proapolipoprotein was given intravenously over 20 minutes. ProapoA-I liposomes (UCB SA, Pharma Sector) were prepared from recombinant human proapoA-I, which was associated with phosphatidylcholine exactly as described previously. One hour before infusion, 40 vials, each containing 100 mg recombinant proapoA-I associated with 125 mg of soybean phosphatidylcholine, were dissolved by the addition of 5 mL NaHCO₃ (20 mmol/L; pH 8.0) per vial. The vials were gently shaken until all particulate material was dissolved, after which the solutions from all vials were mixed and filtered through a 0.22-μm filter. The loss of liposome material is 2% to 5%; the characteristics of this “synthetic HDL” have been described in detail. For the control experiments, an identical protocol was followed, but proapoA-I was excluded from the liposome preparation.

Assays
Measurements of plasma lipoprotein lipids, apoA-I and B, and serum levels of cholesterol precursors and plant sterols were performed repeatedly during the whole study. Fecal neutral sterols (cholesterol, coprostanol, and coprostanone) and fecal bile acids were measured by quantitative gas-liquid chromatography. Complete blood counts, liver and kidney function tests, fasting glucose and insulin, albumin, electrolytes, and thyroid hormones were checked repeatedly during the study and for 15 days thereafter. Sera obtained immediately before infusion and 1, 2, 4, 8, and 15 days thereafter were analyzed for antibodies against recombinant proapoA-I and total Escherichia coli protein by ELISA assays.

Results
The patients were carefully supervised, including continuous ECG and blood pressure monitoring during the infusion. No adverse reactions were observed. All laboratory safety parameters were repeatedly within normal limits before and during follow-up for 15 days.

Two of the patients (S.G. and L.M., who both had coronary disease) had low basal plasma apoA-I levels. In response to the proapoA-I liposome infusion, plasma apoA-I levels increased in all patients (Figure 1A). After 1 hour, total apoA-I concentrations were between 39% and 84% (mean, 64%) above initial values (P<0.005; paired t test). Thereafter, plasma apoA-I levels fell gradually, but they still were

Figure 1. Plasma levels of apoA-I (A) and HDL cholesterol (B) in response to infusion of 4 g proapoA-I/liposome complexes in individual patients.
between 9% and 34% above initial values at 24 hours ($P<0.001$).

The clear increase in apoA-I was accompanied by a less marked elevation of HDL cholesterol levels, as determined after ultracentrifugation (Figure 1B). Peak levels (+8% to +80%; mean, +35%) were observed at 6 hours ($P<0.05$) or 12 hours ($P<0.02$), and in all patients except L.M., HDL cholesterol generally had returned to baseline levels within 24 hours. Separation of lipoproteins by fast protein liquid chromatography confirmed the relatively modest changes in HDL cholesterol. Protein analysis of these lipoprotein fractions by SDS-PAGE demonstrated a moderately increased apoA-I in HDL within the first 12 hours (not shown).

Thus, the drastic expansion of the apoA-I pool was associated with relatively moderate changes in lipoprotein lipid levels. As seen in the Table, there were no significant differences in the mean levels of total, LDL, or HDL cholesterol during the 2 periods when cholesterol elimination was measured. However, when the fecal excretion of bile acids and neutral sterols during 9 days after the infusion was compared with the baseline measurements, remarkable increases were observed in all 4 patients (Figure 2). The mean excretion of bile acids increased by 30% and that of neutral sterols by 39%, corresponding to 2.15 and 4.83 mg/kg·d, respectively (Table). The results thus imply that during >1 week after the infusion, a mean of ~500 mg/d excess cholesterol was being removed. The excretion was not measured for more than 12 days in any individual, so we cannot determine how prolonged this stimulation was. There was no change in serum lathosterol levels in response to the infusion (Table).

To test the possibility that the phospholipid complexes had an independent stimulatory effect on cholesterol excretion, we repeated the study in 2 of the patients, G.G. and L.M., with infusion of liposomes prepared without proapoA-I. In response to the intravenous administration of 5 g phosphatidylcholine, plasma HDL cholesterol levels increased slightly within 1 hour, from 19 to 27 mg/dL in L.M. and from 43 to 50 mg/dL in G.G. This level remained for 12 and 6 hours, respectively, after which lipoprotein levels were similar to baseline. There was no concomitant increase in plasma apoA-I concentration; instead, there was a tendency toward reduced levels (~14% and ~10% after 1 and 6 hours, respectively). Treatment with pure liposomes did not affect the fecal excretion of cholesterol, either as neutral sterols or as bile acids. G.G., whose cholesterol excretion increased by 49% (+12.8 mg · kg$^{-1}$ · d$^{-1}$) when infused with proapoA-I liposomes, displayed only a 2% increase (+0.3 mg · kg$^{-1}$ · d$^{-1}$) after the administration of phosphatidylcholine. Similarly, L.M.'s cholesterol excretion increased 16% (+5.2 mg · kg$^{-1}$ · d$^{-1}$) after proapoA-I complexes and only 2% (+0.8 mg · kg$^{-1}$ · d$^{-1}$) after the control infusion. Safety monitoring did not reveal any abnormalities, and serum lathosterol levels were not affected (not shown).

### Discussion

In this open study, we were able to observe an enhanced cholesterol excretion in response to the infusion of proapoA-I...
liposomes in humans. The dose of proapoA-I administered corresponds to \(\approx75\%\) to 100\% of the calculated plasma apoA-I pool, and the plasma levels reached therefore indicate distribution in the extravascular space as well. In agreement with this contention, the apparent volume of distribution of radioabeled proapoA-I liposomes was calculated to be larger than plasma volume when 8 patients were infused with 1.6 g of an identical preparation of proapoA-I in a previous study.\(^{24}\) The finding of a more sustained HDL cholesterol increase after a lower proapoA-I load in that study may be related to the primary selection of subjects with low initial HDL cholesterol levels. Accordingly, patient L.M. of our study, who had low initial HDL cholesterol, displayed 40\% higher levels for >1 week after the infusion (Figure 1B). Although no detailed kinetic evaluation was performed in the present study, it is of interest to note that the initial rate of lowering of apoA-I clearly was more rapid in the 2 patients with low initial apoA-I levels (Figure 1A). This may reflect a more rapid clearance of the apolipoprotein in those subjects, a phenomenon known to be linked to lower plasma apoA-I levels.\(^{1,2,5,7,38-40}\)

The degree of stimulation of cholesterol excretion in response to proapoA-I infusion observed in our study was surprisingly large. Analyses from 3-day stool collections, as performed here, have been shown to give reliable information on fecal elimination of cholesterol.\(^{35,36}\) The daily variation in fecal steroid excretion was recently reported to be 7.3±1.5\% from the highest and lowest values in 21 subjects.\(^{41}\) We did not study a parallel control group, but the virtually unchanged excretion of cholesterol before and after the infusion of pure liposomes in 2 of the subjects provides a strong argument against the possibility that the change observed in response to proapoA-I was due to technical errors. Obviously, only a limited number of male patients with heterozygous familial hypercholesterolemia were investigated, and generalization of these results therefore has to be done with great caution. Experiments in other groups of subjects, possibly including patients with biliary diversion,\(^{42}\) will be of importance to further confirm our present results.

Two major objections to our interpretation that the increase in cholesterol elimination reflects an enhanced reverse cholesterol transport should be discussed. First, because apoA-I liposomes may interact with liver cell membranes,\(^{16-18}\) they may actually extract cholesterol from the liver and instead create an increased demand for cholesterol in the hepatocyte, resulting in an enhanced biosynthesis of cholesterol,\(^{12,23,43}\) which could result in elevated net excretion of fecal steroids. Although we cannot completely exclude this possibility, we find it less likely because measurements of serum lathosterol concentrations, which provide a good indication of changes in hepatic and overall body cholesterol synthesis,\(^{35,44,45}\) did not show any differences between the 2 study periods. The remote possibility that the apoA-I infusion influenced the intestinal absorption of cholesterol cannot be completely excluded. However, it should be mentioned that serum campesterol, which reflects dietary cholesterol absorption,\(^{32,34}\) was not changed in any of the subjects (not shown).

A second major concern is whether the apolipoprotein moiety of the proapoA-I liposomes is the active factor in promoting cholesterol excretion or whether the soybean phosphatidylcholine liposome component may actually promote reverse cholesterol transport equally well. It is known that phospholipid complexes without apolipoproteins can extract free cholesterol from cellular membranes,\(^{16,17}\) and they may also serve to deliver this cholesterol to hepatocytes. However, in experiments with cholesteryl ester–loaded human macrophages incubated with liposomes containing proapoA-I or only phosphatidylcholine, the former extracted \(\approx70\%\) of the cholesteryl esters, whereas the latter caused a cholesteryl ester egress of only 20\%.\(^ {29}\) To directly address this question, we restudied 2 patients who were given liposomes without proapoA-I. Only minor, if any, changes in cholesterol excretion were observed. Thus, although some changes in cholesterol transfer may well occur in response to apolipoprotein-free liposomes, it is clear that apoA-I plays a major role in promoting the effects observed in the present study.

In conclusion, a pronounced increase in body cholesterol excretion was observed after the intravenous infusion of recombinant proapoA-I liposome complexes. Because the dietary intake of cholesterol was stable and there was no detectable upregulation of body cholesterol synthesis, these results strongly suggest the possibility of stimulating reverse cholesterol transport in humans. Although such estimations are obviously subject to considerable error, it is interesting to note that \(\approx5\) g of excess cholesterol appears to have been removed after the administration of \(4\) g of proapoA-I in liposome form. This may indicate that each apoA-I molecule is utilized several times in cholesterol transport, lending support to the importance of reutilization of HDL apolipoproteins in reverse cholesterol transport.\(^ {5-7,17-22}\) The recent finding of an increased biliary output of cholesterol in mice that overexpress the HDL receptor SR-BI in the liver\(^ {23}\) indicates that this pathway of cholesterol delivery may be an important mechanism explaining the coupling between HDL and cholesterol excretion. Finally, it is of interest to relate the amount of excess cholesterol removed in our patients to the total-body stores of cholesterol, which have been estimated to be \(\approx100\) g.\(^ {46}\) Although we cannot identify the precise source of the excess excreted cholesterol, it is tempting to speculate that repeated treatments with proapoA-I liposomes may actually reduce cholesterol in the arterial wall to some extent. Animal experiments give some reason for optimism for this view, but clinical trials will obviously be necessary to evaluate the antiatherogenic potential of such therapy.

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