Adipose tissue cholesteryl ester transfer protein mRNA in response to probucol treatment: cholesterol and species dependence

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Abstract Probucol treatment results in an increase in plasma concentrations of cholesteryl ester transfer protein (CETP) which may account, in part, for the effects of this agent on plasma concentrations of HDL cholesterol. We have examined the mechanism by which probucol increases plasma CETP and have determined the associated changes in the plasma distribution of high density lipoprotein (HDL) particles. Studies were carried out in nine hypercholesterolemic subjects and five normal volunteers. Probucol treatment resulted in a 31% increase in plasma concentrations of CETP and a 23% decrease in HDL cholesterol ($P < 0.01$). The plasma concentration of LpA-I decreased by 40% ($P < 0.01$) whereas no change occurred in the LpA-I/A-II subclass of HDL. Plasma CETP increased significantly by 1 week of therapy and remained stable over 10 to 14 weeks of therapy. In spite of the significant increase in plasma concentrations of CETP, the abundance of CETP mRNA in peripheral adipose tissue decreased markedly ($P < 0.001$). These results suggested that probucol may alter CETP synthesis in another tissue such as liver or, alternatively, may have other effects on CETP secretion into or catabolism out of the plasma pool. Further studies were carried out in hamsters because, in this species, adipose tissue is a major site and liver is a negligible site for CETP synthesis. Hamsters were fed probucol with or without dietary cholesterol because this species was previously shown to respond to dietary cholesterol with an increase in adipose tissue mRNA levels and in plasma CETP concentrations, thus providing the opportunity to determine whether probucol would alter these parameters independently of the dietary cholesterol effect. When animals were fed a cholesterol-free diet, probucol had no effect on plasma concentrations of HDL-C or CETP or on adipose tissue CETP mRNA abundance. Addition of cholesterol to the diet (0.5% w/w) resulted in significant increases both in plasma CETP and in the level of CETP mRNA in adipose tissue. When probucol was incorporated into the cholesterol-rich diet, there was a further and significant increase in plasma CETP and adipose tissue mRNA abundance and a decrease in HDL cholesterol. The effect of probucol on CETP gene expression may be mediated by alterations in a putative regulatory pool of cellular cholesterol and may, in turn, depend on net transport of cholesterol to and from specific tissues via chylomicrons, low density lipoproteins, or other lipoproteins.—**Quinet, E. M., P. Huerta, D. Nancoo, A. R. Tall, Y. L. Marcel, and R. McPherson.** Adipose tissue cholesteryl ester transfer protein mRNA in response to probucol treatment: cholesterol and species dependence. *J. Lipid Res.* 1993. **34:** 845–852.

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Cholesteryl ester transfer protein (CETP) is a 74 kD glycoprotein that mediates the molar exchange of cholesteryl ester (CE) and triglycerides (TG) between apolipoprotein (apo) A-I- and apoB-containing lipoproteins (1). This process results in the net transfer of CE from HDL to triglyceride-rich lipoprotein remnants, and kinetic studies in species with significant levels of CETP in plasma, specifically humans and the rabbit, suggest that CETP mediates the major route for the return of HDL cholesterol to the liver (2, 3). The beneficial or detrimental effect of the CETP-mediated transfer process on atherogenesis is likely to be dependent on the efficacy of hepatic clearance mechanisms for VLDL remnants.

Previous studies have demonstrated that CETP messenger RNA (mRNA) levels in liver and adipose tissue and CETP mass in plasma are increased by dietary cholesterol in humans and other species (4–6). Increased CETP synthesis in response to increased tissue cholesterol may reflect a requirement for this protein in redistributing cholesterol between various tissues or organs. Probucol treatment also results in increased plasma levels of CETP (7) and in increased isotopic cholesteryl ester transfer activity, albeit under conditions designed to

Abbreviations: CE, cholesteryl ester; CETP, cholesteryl ester transfer protein; TG, triglyceride; HDL, high density lipoprotein; LDL, low density lipoprotein; C, cholesterol.

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minimize the effects of donor and acceptor lipoproteins (8). The change in plasma CETP mass may explain, in part, the effect of this agent on HDL concentration and composition. There are no previous reports on the effect of probucol on apoA-I with A-II (LpA-I/A-II) HDL particles versus apoA-I without apoA-II (LpA-I) particles but if the decrease in HDL is due, in part, to increased CETP activity, one might expect a greater decrement in LpA-I as these particles apparently transport 80% or more of plasma CETP (9, 10).

The mechanism of the probucol effect on plasma CETP is not known and could represent increased CETP synthesis in the liver or other tissues or delayed catabolism of CETP out of the plasma pool due to alterations in the small α_2-migrating HDL subspecies which transports CETP in plasma (11). We have determined the effect of probucol on plasma concentrations of CETP and on levels of CETP mRNA in adipose tissue in humans and in hamsters. These results demonstrate that probucol increases adipose tissue CETP mRNA in the presence, but not in the absence, of dietary cholesterol, suggesting that, in the hamster, the probucol effect is mediated by alterations in a specific cellular pool of cholesterol that regulates CETP gene transcription.

METHODS

Hypercholesterolemic Subjects

Nine male and female patients registered with the McGill Lipid Clinic and having primary polygenic hypercholesterolemia were enrolled in the study. All participants were in good general health with normal serum levels of glucose, creatinine, hepatic transaminases, electrolytes, and calcium. Baseline and treatment electrocardiograms revealed no conduction abnormalities. Subjects were stabilized on an American Heart Association Step I diet, containing less than 250 mg of cholesterol per day. Lipid-lowering agents (lovastatin, cholestyramine) were withdrawn 2 months prior to the start of the study. Other medications were limited to calcium channel blockers or ACE inhibitors for the treatment of hypertension (four patients) and these were continued throughout the study. Hypercholesterolemic patients were treated with probucol (500 mg b.i.d.) for a 14-week period. Compliance was assessed by tablet count. Blood was collected on ice in EDTA-containing tubes (1 mg/ml) after a 14-h overnight fast under standardized conditions of posture with minimal use of a tourniquet. Plasma samples were collected at baseline and after 4, 8, and 14 weeks of treatment for plasma lipoproteins, apolipoproteins, and CETP.

Normal subjects

Five normal male subjects on no medication, ingesting low-cholesterol diets (< 250 mg/day) and with serum normal biochemistry and TSH levels were treated with probucol (500 mg b.i.d.) for an 8-week period and fasting plasma was collected as described above on a weekly basis to determine the time-course effects of this agent on plasma CETP and HDL composition.

Adipose tissue biopsies

Adipose tissue biopsies were collected from three normal and seven hyperlipidemic subjects at baseline and after 8 weeks (normal subjects) or 14 weeks (hyperlipidemic subjects) of treatment. Adipose biopsies (5 g) were obtained after local lidocaine anesthesia from a periumbilical skinfold using a 4-mm liposuction canula. Samples were collected on dry ice and transferred immediately to liquid nitrogen.

Informed consent

The study was approved by the Institutional Review Board of the Royal Victoria Hospital. Written informed consent was obtained from all participants.

Hamsters

Male Golden Syrian hamsters weighing 100–120 g were purchased from Charles River Laboratories. Animals were randomized to one of four diets (11 animals per group). Diets were as follows: A) Purina rodent chow; B) Purina rodent chow plus 0.5% cholesterol (w/w); C) Purina rodent chow plus 0.5% probucol (w/w); and D) Purina rodent chow plus 0.5% cholesterol (w/w) and 0.5% probucol (w/w). Diets were prepared and pelleted by Purina Mills, Richmond, IN. Probucol was the generous gift from Marion Merrell Dow Research Institute, Cincinnati, OH. Animals were fed ad libitum for a 6-week period and anesthetized with sodium pentobarbital after an overnight fast. Animals were exsanguinated by cardiac puncture and 5-g portions of liver and epididymal adipose tissue were collected and frozen immediately in liquid nitrogen.

Plasma lipoproteins, apolipoproteins, and CETP

Total cholesterol, triglycerides, and HDL-C were measured by the Lipid Research Clinics protocol using automated methods, standardized with the Centers for Disease Control in Atlanta (12). VLDL ($d < 1.006$ g/ml) was isolated ultracentrifugally. Plasma apoA-I and B were determined by immunoelectrophoresis using the Sebia gels. LpA-I and LpA-I/A-II were determined by differential immunoassay (13) using the Sebia gels (Sebia, France). CETP (11) and apoE (14) were determined by radioimmunoassay as described previously. We have previously demonstrated that in normal and hyperlipidemic subjects CETP mass in plasma correlates well with isotopic cholesteryl ester transfer activity measured in vitro ($r = 0.72$, $P < 0.05$) (15). The between-run coefficients of variation between replicate samples were 1.8% for total

cholesterol, 3.6% for triglyceride, 3.4% for HDL cholesterol, 3.0% for apoA-I, 3.5% for apoB, and 3.9% for CETP.

Adipose tissue CETP mRNA levels

Total cellular RNA was extracted using the acidic guanidinium isocyanate technique as described by Chomczynski and Sacchi (16). Northern transfer of poly A + mRNA was performed to verify the size of the CETP message. The abundance of CETP mRNA was determined by a solution hybridization ribonuclease protection assay (4, 17). Using conditions described previously, 30 μg of test total RNA from human adipose tissue was hybridized to a human antisense RNA probe prepared from the human CETP cDNA (289 bp fragment, nucleotides 674–957) subcloned into pGEM4Z. After 16–18 h of hybridization at 48°C, samples were digested by RNAse T2 for 2 h at 30°C (4) and [^{32}P]RNA-RNA hybrids were analyzed on 6% polyacrylamide-urea sequencing gels. Protected fragments were visualized by autoradiography and quantitated by densitometry. RNA mass was determined by comparison with a standard curve of CETP cRNA hybridized simultaneously. For this purpose, sense strand RNA was synthesized by in vitro transcription and its mass was quantitated precisely by standard methods using [^{3}H]NTP incorporation.

Statistical analyses

Paired *t*-tests were used to compare differences in biochemical variables between the control and probucol treatment periods. Analysis of variance was performed to compare responses in animals treated with dietary cholesterol. Regression analyses were performed using the SAS statistical package (18, 19).

TABLE 1. Plasma lipoprotein responses to probucol in hypercholesterolemic subjects

Lipoprotein	Baseline	Treatment
	mmol/l	
TC	7.0 ± 0.8	6.2 ± 9[a]
TG	1.9 ± 0.8	2.0 ± 1.5
VLDL-C	0.62 ± 0.33	0.55 ± 0.44
VLDL-TG	1.27 ± 0.77	1.26 ± 1.26
LDL-C	5.30 ± 0.89	4.81 ± 0.97[a]
LDL-TG	0.52 ± 0.14	0.56 ± 0.24
HDL-C	1.09 ± 0.16	0.84 ± 0.21[b]
HDL-TG	0.19 ± 0.06	0.16 ± 0.07

Data are expressed as means ± SD for nine subjects; treatment period was 14 weeks.
[a] $P < 0.05$.
[b] $P < 0.01$.

TABLE 2. Plasma concentrations of LpA-I, LpA-I/A-II, CETP, and apolipoprotein E before and after probucol treatment in humans

Variable	Baseline	Probucol Treatment
	mg/dl	
LpA-I	41.6 ± 18.0	24.9 ± 7.2[a]
LpA-I/A-II	75.0 ± 27.1	84.3 ± 18.4
CETP	0.207 ± 0.055	0.269 ± 0.083[b]
ApoE	11.3 ± 7.6	19.3 ± 12.0[b]

Five normal and nine hypercholesterolemic subjects were studied before and after 8 weeks (normal) or 14 weeks (hyperlipidemic subjects) of probucol treatment. Data are expressed as means ± SD.
[a] $P \leq 0.01$.
[b] $P \leq 0.001$.

RESULTS

Compliance

All normal and hyperlipidemic subjects completed the study uneventfully. Compliance, as assessed by tablet count was > 85% for all subjects. Side-effects were limited to complaints of increased bowel frequency in three subjects.

Plasma lipoproteins

The hyperlipoproteinemic subjects treated with probucol demonstrated changes in lipoproteins similar to those previously reported (7). HDL-C decreased by 23% and LDL-C decreased by 9% (**Table 1**). These changes occurred promptly and no further significant changes were noted in any lipoprotein fraction or in CETP between 4 and 14 weeks of treatment.

LpA-I and LpA-I/A-II

The percentage changes in these variables, as well as CETP and apoE, were similar for the normal and hyperlipidemic subjects, hence the data for normal and hyperlipidemic subjects was pooled. For all subjects combined, there was a 40% decrease in the plasma concentration of LpA-I particles with no significant change occurring in LpA-I/A-II particles (**Table 2**).

CETP and apoE

As reported previously (7) CETP and apoE increased significantly, by 31% and 67%, respectively, for 14 subjects after probucol therapy (Table 2).

Time course changes in CETP and HDL

In the five normal subjects, in whom early time-course changes in plasma lipoproteins and CETP in response to probucol were studied, plasma CETP concentrations increased significantly after only 1 week of therapy with a

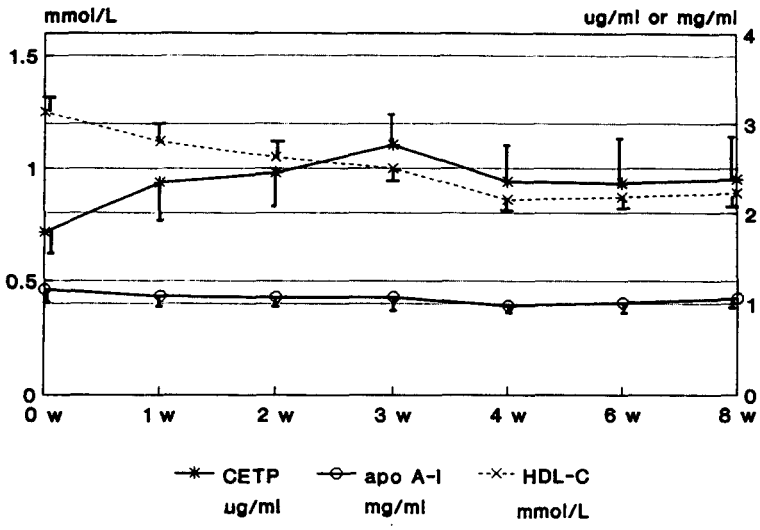

Fig. 1. Early time-course changes in CETP, apoA-I and HDL cholesterol in five normal subjects treated with probucol. Changes in plasma concentration of CETP and HDL-C were significant ($P < 0.05$) at each of 1, 2, 3, 4, 6, and 8 weeks of treatment. The decrease in apoA-I was significant at weeks 2, 4, and 6 only ($P < 0.05$).

maximum plasma level evident at 3 weeks of treatment. HDL-C decreased in a concomitant fashion reaching a nadir at 4 weeks of treatment and then stabilized (**Fig. 1**).

Adipose tissue mRNA levels

Despite the highly significant increase in plasma concentrations of CETP in both normal and hyperlipidemic subjects, levels of CETP mRNA in adipose tissue decreased profoundly in all but one subject during probucol therapy (**Table 3**).

Plasma lipids and CETP and adipose tissue CETP mRNA in hamsters

Plasma lipid levels in hamsters fed chow with or without probucol and/or cholesterol are shown in **Table 4**.

TABLE 3. Adipose tissue CETP mRNA levels in normal and hypercholesterolemic subjects before and after probucol treatment

Subject	Baseline	Probucol[a]
	pg CETP mRNA/μg total RNA	
DS[b]	0.686	0.036
BG[b]	0.273	0.121
HM[b]	0.394	0.118
NH[b]	0.293	0.097
RS[b]	0.084	0.032
GD[b]	0.180	0.025
LD[b]	0.164	0.195
NS[c]	0.839	0.487
DB[c]	0.140	0.022
FC[c]	0.238	0.161
Mean	0.329	0.129[d]
SD	0.247	0.139

[a] Results after 8 weeks (normal subjects) or 14 weeks (hyperlipidemic subjects) of treatment with probucol.
[b] Hyperlipoproteinemic patient.
[c] Normal subject.
[d] $P < 0.01$.

For animals on all four diets, there was a significant relationship between adipose tissue CETP mRNA abundance and plasma concentrations of CETP ($r = 0.76$, $P < 0.01$; **Fig. 2**). The plasma CETP and HDL cholesterol responses to probucol in the hamster differed markedly in animals fed diets with or without cholesterol.

Cholesterol-free diet

When hamsters were fed chow without cholesterol, probucol had no significant effects on plasma lipoproteins or CETP (Table 4 and **Fig. 3A**). Similarly, there were no differences in CETP mRNA levels in adipose tissue in animals fed chow with or without the addition of probucol (Fig. 3B).

Cholesterol-rich diet

Addition of cholesterol to the chow diet resulted in significant increases in total cholesterol, triglycerides, HDL-C, and in CETP as compared to the cholesterol-free diet as reported previously (4–6). Animals fed a cholesterol-rich diet plus probucol demonstrated further increases in plasma CETP and this increase in CETP was associated with a significant decrease in HDL-C as compared to animals fed the cholesterol-rich diet without probucol. Similarly, in adipose tissue, probucol significantly augmented the increase in CETP mRNA observed in animals fed cholesterol alone (Fig. 3A). Linear regression analyses demonstrated that there were significant inverse correlations between plasma CETP and HDL-C ($r = -0.47$, $P < 0.05$) (**Fig. 4A**) and between adipose tissue CETP mRNA and HDL-C ($r = -0.73$, $P < 0.01$) (Fig. 4B) for animals fed a cholesterol-rich diet with or without probucol. When animals on all four diets were included in the linear regression analysis, the negative relationship between plasma CETP and HDL-C disappeared due to the positive effects of cholesterol feeding on both of these variables.

TABLE 4. Plasma lipid responses to probucol and/or dietary cholesterol in hamsters

Light	Chow Diet (11)	Chow Diet + Probucol (11)	0.5% Chol (10)	0.5% Cholesterol + Probucol (9)
			mmol/l	
TC	2.8 ± 0.3	3.1 ± 0.3[a]	11.8 ± 2.0[a]	10.7 ± 1.5[a]
TG	2.3 ± 0.3	3.0 ± 0.3[a]	7.7 ± 2.9[a]	7.1 ± 2.0[a]
HDL-Chol	1.39 ± 0.09	1.36 ± 0.10	2.25 ± 0.15[a]	1.65 ± 0.18[a,b]

Data are expressed as means ± SD; number of animals in each group is given in parentheses.
[a] Significantly different from chow diet ($P < 0.05$).
[b] Significantly different from 0.5% cholesterol diet.

DISCUSSION

The plasma concentration and activity of CETP influences steady state concentrations of HDL in various situations. In human genetic CETP deficiency, HDL size and cholesterol content are markedly increased (20, 21). We have demonstrated that treatment of chylomicronemic patients with a low-fat diet effectively decreases plasma CETP and increases HDL-C (15). The present study confirms our previous report that probucol treatment significantly increases plasma concentrations of CETP (7). Probucol has been shown to reduce apoA-I production in human apoA-I transgenic mice (22) but the effect on plasma concentration and activity of CETP may also contribute to the reduction in HDL size and cholesterol content associated with this medication (7, 8). We have demonstrated that the increase in plasma CETP associated with probucol treatment occurs promptly (by 1 week of treatment) and the decrease in HDL cholesterol is concomitant.

Cheung and colleagues (10) reported that CETP in plasma is mainly associated with apoA-I-only particles and recent in vitro studies using recombinant phospholipid-rich HDL particles have demonstrated that apoA-I-only particles are preferentially converted to smaller particles in the presence of CETP, whereas incorporation of apoA-II into recombinant HDL particles inhibited the conversion, in the presence of CETP, to a smaller particle (9). We have demonstrated that the reduction in plasma HDL concentrations in patients treated with probucol is associated with a 40% reduction in the plasma concentration of LpA-I particles with no change occurring in the plasma concentration of LpA-I/A-II particles. These results are consistent with the hypothesis that, in the case of cholesteryl ester transfer, there is also a preferential interaction between CETP and apoA-I-only particles.

Despite the highly significant increase in plasma concentrations of CETP in response to probucol treatment in normal and hypercholesterolemic subjects, there was a

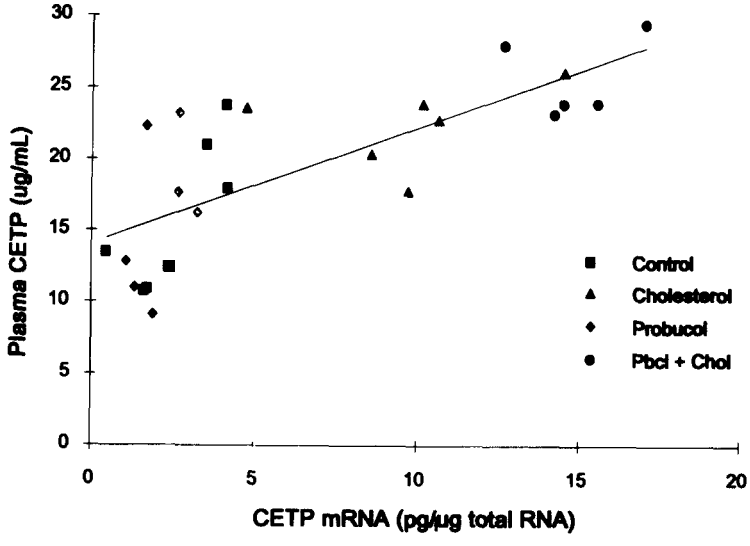

Fig. 2. Correlation between adipose tissue CETP mRNA abundance and plasma concentration of CETP in hamsters. Data points represent single measurements of adipose tissue mRNA and plasma CETP for 25 animals fed diets with or without cholesterol and/or probucol ($r = +0.76$, $P < 0.0001$).

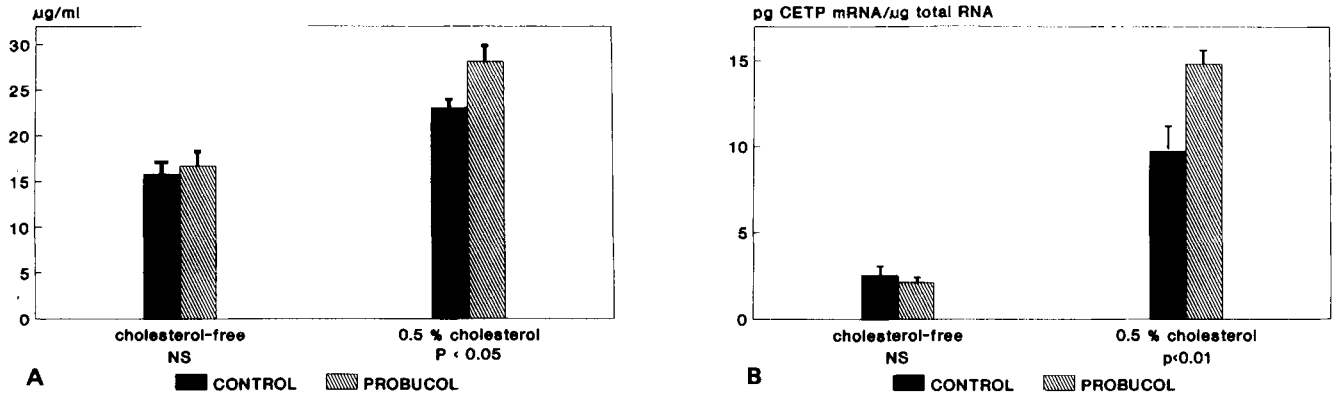

Fig. 3. A: Effect of dietary cholesterol and/or probucol on plasma CETP concentrations in hamsters. Plasma CETP concentrations were higher in animals fed cholesterol versus chow-fed animals and higher in animals fed probucol plus cholesterol as compared to animals fed probucol alone ($P < 0.02$). Probucol significantly increased plasma concentrations of CETP when the diet contained cholesterol ($P < 0.05$) but had no significant effect on plasma CETP levels in animals fed a low cholesterol chow diet; numbers of animals analyzed in each group are as follows: 11 control/cholesterol-free, 11 probucol/cholesterol-free, 10 control/cholesterol-fed, and 9 probucol/cholesterol-fed. Data are expressed as mean and SEM. B: Effect of dietary cholesterol and/or probucol on adipose CETP mRNA levels in hamsters. Inclusion of cholesterol in the diet resulted in increases in adipose tissue CETP mRNA whether or not the diet contained probucol ($P < 0.01$). Probucol significantly increased CETP mRNA levels in adipose tissue only when the background diet contained cholesterol ($P < 0.01$); numbers of animals analyzed in each group are as follows: 7 control/cholesterol-free, 7 probucol/cholesterol-free, 6 control/cholesterol-fed, and 5 probucol/cholesterol-fed. Data are expressed as mean and SEM.

marked decrease in the abundance of CETP mRNA in peripheral adipose tissue. There are several possible explanations for this observation. Adipose tissue is only one important site of CETP synthesis in humans and other tissues, such as liver, are also major contributors to CETP production. CETP mRNA levels are induced by dietary cholesterol (4, 5, 23) and probucol has been reported to increase hepatic clearance of LDL by a non-LDL receptor-mediated pathway (24). Thus, it is possible that, probucol treatment in humans results in a redistribution of tissue cholesterol with a decrease in the adipose tissue cholesterol pool, which is a putative regulator of CETP synthesis, and a reciprocal alteration in cellular sterol and CETP synthesis in another tissue, such as liver. Alternatively, probucol may have direct effects on the secretion of CETP into plasma or, due to alterations in the size and composition of HDL, effects on the catabolism of CETP out of the plasma pool.

To clarify whether the effects of probucol on plasma levels of CETP were due to alteration in CETP mRNA production, we proceeded to determine the effects of probucol on plasma CETP and CETP mRNA in adipose tissue in the hamster. The hamster was chosen as an animal model because there is virtually no CETP mRNA detected in the liver and a major site of CETP synthesis is adipose tissue (25). As we had demonstrated in previous

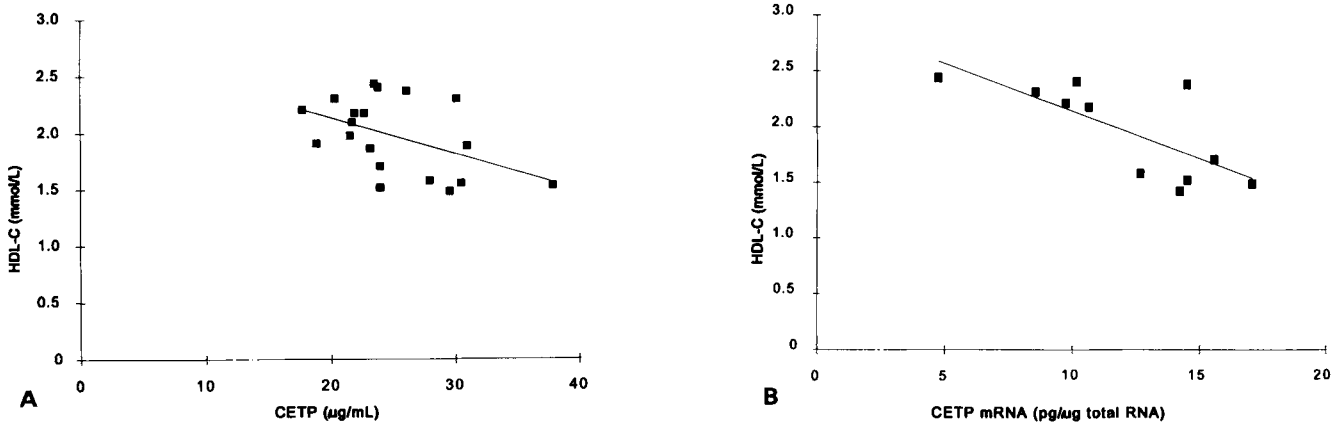

Fig. 4. Correlation between plasma CETP and plasma HDL cholesterol in hamsters fed cholesterol-containing diets. Data points represent a single measurement of plasma CETP and plasma HDL cholesterol in animals consuming cholesterol-containing diets with and without probucol (n = 10 cholesterol-fed and 9 probucol + cholesterol; $r = -0.47$, $P < 0.05$). B: Correlation between adipose tissue mRNA abundance and plasma HDL cholesterol in hamsters fed cholesterol-containing diets. Data points represent a single measurement of adipose tissue CETP mRNA and plasma HDL-C in animals fed cholesterol-containing diets with or without probucol (n = 6 cholesterol-fed and 5 probucol + cholesterol; $r = -0.73$, $P < 0.01$).

studies that cholesterol feeding results in increases in CETP mRNA in adipose tissue and in CETP levels in plasma in humans and animals (4–6, 26, 27), we tested the effects of probucol feeding with and without the simultaneous incorporation of cholesterol into the diet to determine whether any probucol effect was independent of that of dietary cholesterol.

The results, in agreement with previous studies (4), show that plasma CETP concentration is strongly correlated with the abundance of CETP mRNA in adipose tissue (Fig. 2). Furthermore, in the context of cholesterol feeding, there was a significant inverse correlation between plasma CETP concentration and HDL cholesterol and an even stronger inverse relationship between adipose tissue CETP mRNA abundance and HDL cholesterol concentration, suggesting that CETP synthesized by adipocytes may play a role in HDL remodelling in an extravascular compartment. These studies clearly demonstrate that in an animal in which adipose tissue is a major site of CETP synthesis (25), probucol increases CETP mRNA levels, increases plasma concentrations of CETP, and decreases plasma concentrations of HDL-C but only in the presence of dietary cholesterol. Furthermore, these observations suggest that the probucol-mediated regulation of CETP gene transcription in peripheral adipose tissue in the hamster is cholesterol-dependent. Probucol is very lipid-soluble and is readily incorporated into adipose tissue, where it may alter the specific cellular pool of cholesterol or oxysterol which is a putative regulator of CETP gene transcription.

Adipose tissue is an important cholesterol storage organ in humans and contains approximately 2 mg of cholesterol per gram of adipose tissue (28), representing 20–50% of exchangeable cholesterol in normal and obese subjects, respectively (29). Furthermore, the cholesterol content of adipose tissue increases in response to cholesterol feeding (30), as does CETP mRNA abundance. Regulation of adipose tissue CETP synthesis by cellular sterol may play a role in tissue cholesterol trafficking.

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