Higher Plasma Lipid Transfer Protein Activities and Unfavorable Lipoprotein Changes in Cigarette-Smoking Men

Robin P.F. Dullaart, Klaas Hoogenberg, Bert D. Dikkeschei, Arie van Tol

Abstract The mechanisms responsible for the atherogenic lipoprotein changes associated with cigarette smoking are largely unknown. Lecithin:cholesterol acyltransferase (LCAT) and cholesteryl ester transfer protein (CETP) are key factors in the esterification of plasma cholesterol and the transfer of cholesteryl ester from high-density lipoproteins (HDLs) toward very-low- and low-density lipoproteins (VLDLs+LDLs). Another transfer factor, phospholipid transfer protein (PLTP), recently has been shown to be involved in the interconversion of HDL particles in vitro, but its physiological function is not yet clear. We measured the activities of LCAT, CETP (as cholesteryl ester exchange activity), and PLTP using exogenous substrate assays as well as lipoprotein profiles in the plasma of 21 normolipidemic cigarette-smoking men (total plasma cholesterol below 6.5 mmol/L and triglyceride below 2.5 mmol/L) and 21 individually matched nonsmoking control subjects. HDL cholesterol, HDL cholesteryl ester, and plasma apolipoprotein A-I levels were lower in the smokers than in the control subjects (P<.05 for all parameters). Median plasma CETP activity was 18% higher (P<.02) and median plasma PLTP activity was 8% higher (P<.05) in the smokers compared with the nonsmokers. LCAT activity was not different between the groups. HDL cholesteryl ester concentration was positively related to LCAT activity in control subjects but not in smokers. By contrast, there was an inverse relation of CETP activity with HDL cholesteryl ester in smokers but not in nonsmokers. Multiple regression analysis demonstrated that the lowering effect of smoking on HDL cholesteryl ester could be explained by its influence on CETP activity. In both groups VLDL+LDL cholesteryl ester levels were positively related to CETP activity as well as to PLTP activity. Thus, the higher the levels of CETP and PLTP activity in smokers are, the higher are cholesteryl ester concentrations in VLDL+LDL and the lower in HDL. This case-control study suggests that higher levels of lipid transfer protein activities may provide a mechanism that contributes to unfavorable lipoprotein changes associated with cigarette smoking.

Key Words: • cigarette smoking • phospholipid transfer protein • lipoproteins • cholesteryl ester transfer protein • lecithin:cholesterol acyltransferase

Cigarette smoking is associated with higher levels of very-low- and low-density lipoprotein (VLDL+LDL) cholesterol and lower levels of high-density lipoprotein (HDL) cholesterol. Such unfavorable lipoprotein alterations can explain in part the increased risk of cardiovascular disease among smokers. Of other factors involved in lipoprotein metabolism, lecithin:cholesterol acyltransferase (LCAT, EC 2.3.1.43) and cholesteryl ester transfer protein (CETP) play key roles in the esterification of cholesterol in plasma and the subsequent transfer of cholesteryl ester (CE) toward apolipoprotein (apo) B-containing lipoproteins. High levels of CETP activity have been previously documented in primary and secondary hyperlipidemia. Furthermore, HDL cholesterol is extremely elevated in cases of familial CETP deficiency. Apart from CETP, another lipid transfer factor, phospholipid transfer protein (PLTP), specifically catalyzes the transfer of phospholipids in plasma. Although its physiological function is not well defined, PLTP is involved in regulating HDL particle size in vitro. The mechanisms responsible for the unfavorable lipoprotein levels in smokers still are not precisely understood. Previous studies did not demonstrate significant alterations in the plasma cholesterol esterification rate in smokers. CETP activity was found to be elevated in cigarette-smoking men with insulin-dependent diabetes mellitus, but it is unknown whether such abnormalities are present in smoking subjects without other cardiovascular risk factors.

We hypothesized that alterations in plasma lipid transfer protein activities could play a role in the development of unfavorable lipoprotein changes in smokers. In this case-control study we measured plasma CETP, PLTP, and LCAT activity in carefully matched healthy smokers and nonsmokers and related these factors to lipoprotein levels.

Methods

Subjects

Male subjects, aged 21 to 60 years, were recruited from hospital personnel and consented to the procedure after explanation of the purpose of the study. To be included in the study, subjects had to have fasting plasma total cholesterol (TC) and triglyceride (TG) levels less than 6.5 mmol/L and 2.5 mmol/L, respectively. The men were nonobese (body mass index [BMI] below 27 kg/m²), had a normal blood pressure (systolic blood pressure below 140 mm Hg and diastolic blood
pressure below 95 mm Hg), and did not use any medication. Routine thyroid and liver function tests showed no abnormalities, and the subjects did not suffer from diabetes mellitus. Smokers were eligible if they smoked at least 5 cigarettes daily. Twenty-one smokers were individually matched for age and BMI with a similar number of nonsmokers. The smokers currently smoked a median of 15 (interquartile range [IR], 10 to 25) cigarettes a day and had been smoking for 11 (IR, 8 to 17) pack-years. Thirteen of the nonsmokers had never smoked, and 8 of them had stopped smoking for at least 2 years before the study (former smokers). Both groups were closely comparable with respect to age (33±8 versus 35±11 years for smokers and nonsmokers), BMI (23.1±1.7 versus 22.8±1.4 kg/m$^2$), and waist-to-hip circumference ratio (0.86±0.09 versus 0.84±0.05, measured as the ratio of the smallest girth between the rib cage and iliac crest and the largest girth between the waist and thigh). Blood pressure and fasting blood glucose levels were similar in the groups (not shown). A questionnaire showed no significant differences in physical activity between the groups. The smokers consumed slightly more alcohol than the nonsmokers (2 [IR, 1 to 2] versus 1 [IR, 0.5 to 1.5] drinks per day, *P<.05 by unpaired Wilcoxon's test). The subjects were studied after a 12-hour fast. The smokers refrained from smoking for at least 60 minutes before blood sampling, since cigarette smoking may acutely lower HDL cholesterol.

**Laboratory Measurements**

Venous blood was collected into tubes containing EDTA (final concentration, 1.5 mg/mL) and was placed on ice immediately. Plasma was separated within 30 minutes by centrifugation at 3000 rpm for 15 minutes at 4°C. Samples were frozen at −20°C until analyzed.

The plasma activities of LCAT, CETP, and PLTP were determined using excess exogenous substrates as previously described. CE exchange activity (designated CETP activity) was measured in the supernatant fraction of each plasma sample after removal of endogenous VLDL+LDL by phosphotungstate/MgCl$_2$ precipitation. The isotope assay detects the exchange of radioactive CE between exogenous (1-[$^3$H]oleate)-CE-labeled LDL and excess unlabeled pooled normal HDL. LCAT was completely inhibited with dithiothreitol (2-nitrobenzoic acid) (final concentration, 2 mmol/L). The CETP activity level obtained by this method is well correlated with CETP mass. PLTP activity was assayed in each plasma sample using a phospholipid vesicle-HDL system, exactly according to a recently described procedure. The method is specific for PLTP activity and is not influenced by the phospholipid transfer-promoting properties of CETP. The assays were each performed in duplicate using single batches of substrates. The intra-assay coefficients of variation were 4.5%, 2.7%, and 3.5% for LCAT, CETP, and PLTP, respectively. The measured activities reflect the activity of the enzyme and lipid transfer proteins as such and are independent of the endogenous lipoproteins present in each plasma. The activities of LCAT, CETP, and PLTP were related to the activities of these factors measured in human pooled plasma that was included in each run. LCAT activity is expressed in nanomoles of esterified cholesterol per milliliter of plasma per hour, CETP activity is expressed in nanomoles of cholesteryl ester per milliliter of plasma per hour, and PLTP activity is expressed in micromoles of phosphatidylcholine per milliliter of plasma per hour.

Lipid levels were measured in plasma and in the HDL-containing supernatant fraction after removal of apo B-containing lipoproteins by precipitation with polyethylene glycol-6000 as described. VLDL+LDL lipids were calculated as the difference between plasma and the HDL-containing supernatant. TC was measured by gas chromatography, and free cholesterol (FC) was measured by a modification of this method in which the hydrolysis step was omitted. CE was calculated as the difference between TC and FC. TG was measured enzymatically. Phospholipids were measured according to Zilversmit. Apo A-I and apo B were measured by immunoturbidimetry using commercially available kits (Boehringer Mannheim, catalog Nos. 726478 and 726494, respectively). Lipoprotein(a) was determined by enzyme-linked immunosorbent assay (Biopool Tint Elize, catalog No. 610225).

**Statistical Analysis**

Values are expressed as mean±SD or as median (IR). Between-group comparisons were carried out using Student's *t* tests and Wilcoxon's test for unpaired observations where appropriate. Differences in LCAT, CETP, and PLTP activity are expressed as percent difference (median, 95% confidence interval [CI]). Linear regression analysis was used to analyze relations between data. Multiple regression analysis was performed to evaluate the independent relations between parameters. A two-sided probability value less than .05 was considered to be significant.

**Results**

Plasma LCAT, CETP, and PLTP activities are shown in Fig 1. Plasma LCAT activity was not significantly
different between smokers and nonsmokers: 49 (IR, 41 to 52) versus 47 (IR, 45 to 55) nmol esterified cholesterol per milliliter plasma per hour, respectively; median difference -4% (95% CI, -15% to 10%) (Fig 1A). Both plasma CETP and PLTP activities were higher in smokers versus nonsmokers: 100 (IR, 89 to 114) versus 85 (IR, 78 to 95) nmol CE per milliliter plasma per hour, respectively, P<.02, for CETP activity (Fig 1B) and 6.0 (IR, 5.5 to 6.4) versus 5.5 (IR, 5.0 to 6.0) nmol phosphatidylcholine per milliliter plasma per hour, respectively, P<.05 for PLTP activity (Fig 1C). The median differences in plasma CETP and PLTP activities between smokers and nonsmokers were 18% (95% CI, 4% to 31%) and 8% (95% CI, 0.1% to 18%), respectively. Among nonsmokers there were no differences in lipid transfer protein activities between the former smokers and men who had never smoked (P>.10).

Mean HDL cholesterol and plasma apo A-I levels were lower, whereas the ratio of plasma TC to HDL cholesterol was higher in smokers versus nonsmokers (Table). Plasma TC, TG, and apo B levels were comparable between the groups. The distribution of plasma lipoprotein(a) was similar in smokers and nonsmokers. The reduction in HDL cholesterol in the smokers coincided with a decrease in HDL-CE concentration (0.83±0.12 versus 0.91±0.13 nmol/L in smokers versus nonsmokers, P=.05), as well as in HDL-CE content (33.4±3.2 versus 36.1±4.8 mol % of total lipids, P<.05). No significant differences in the HDL-FC, TG, and phospholipid content were present (data not shown).

Remarkable differences in the relations of HDL-CE to LCAT and CETP activity were observed between smokers and nonsmokers (Fig 2A and 2B). In nonsmokers HDL-CE was positively related to LCAT activity (r=0.69, P<.001) but not to CETP activity (r=0.11, NS). In contrast, HDL-CE was not positively related to plasma LCAT activity in the smokers (r=0.58, P<.01). In this group an inverse relation was present between CETP activity and HDL-CE (r=0.51, P<.02), indicating that the higher the level of CETP activity, the lower the level of HDL-CE. There was no relation of PLTP activity to HDL-CE or to HDL phospholipids in any of the groups (all P>.10). Multiple regression analysis was performed in the combined groups (n=42) to evaluate the independent relations of HDL-CE to LCAT activity, CETP activity, and smoking. HDL-CE was inversely related to CETP activity (P<.005) and positively related to LCAT activity (P<.05, multiple r=.52). The contribution of smoking, introduced in the model either as a categorical covariate (smoking or nonsmoking, P=.44) or as the number of cigarettes smoked daily (P=.25), was not significant. Thus, there was no demonstrable lowering effect of smoking on the level of HDL-CE, independently of its influence on CETP activity. In addition, the relation of plasma TG with HDL-CE was not significant (P=.15). As shown in Fig 3A, VLDL+LDL-CE was positively related to CETP activity both in the smokers (r=0.58, P<.01) and in the nonsmokers (r=0.54, P<.02). VLDL+LDL-CE was also correlated with PLTP activity (smokers: r=0.66, P<.01; nonsmokers: r=0.51, P<.02) (Fig 3B). CETP and PLTP activities were interrelated in the smokers (r=0.61, P<.01), but no further relations among LCAT, CETP, and PLTP activities were observed (all P>.10). Neither LCAT nor lipid transfer protein activities were significantly correlated with alcohol intake (all P>.10).

Discussion

The present study showed higher plasma activities of CETP and PLTP in healthy smokers compared with nonsmokers. Plasma HDL-CE was inversely related to
CETP activity in smokers, whereas VLDL+LDL-CE levels were positively related to the activities of both CETP and PLTP. Moreover, we could not demonstrate a lowering effect of smoking on HDL-CE independently of its influence on CETP activity. These findings suggest that alterations in plasma lipid transfer protein activities may contribute to unfavorable lipoprotein changes associated with smoking.

To avoid confounding influences of hyperlipidemia and adiposity on the activities of LCAT and lipid transfer proteins,6,9,10,20,23 only subjects with normal weight and without hyperlipidemia participated. Despite this selection, HDL cholesterol and plasma apo A-I levels were lower and the ratio of plasma TC to HDL cholesterol was higher in smokers than in nonsmokers. A recent meta-analysis has demonstrated accordingly that HDL cholesterol is about 6% lower in smokers.1 Other studies have shown that HDL cholesterol is decreased.16,24 The diminished HDL-CE content also points to an abnormal HDL composition. There was considerable overlap in individual HDL-CE as well as in plasma CETP activity levels between smokers and nonsmokers. Thus, in otherwise normolipidemic men the effects of smoking on both HDL-CE and CETP activity are modest. The higher plasma CETP activity level in healthy cigarette-smoking subjects is in accordance with observations in insulin-dependent diabetic patients determined using similar analytical methods.17

The present findings disagree with a recent study that showed slightly lower plasma CETP activity levels in smokers determined by assay with radiolabeled HDL₃ and a mixture of VLDL and LDL.²⁴ In contrast to our study, men and women as well as obese subjects were included in that report, and smokers were not individually matched for BMI and sex with nonsmokers.

Experimental evidence underscores the possibility that higher levels of CETP activity can contribute to lower HDL-CE concentrations in smokers. Administration of human CETP decreases HDL-CE in the rat,²⁵ whereas immunologic inhibition of CETP increases HDL-CE and HDL particle size in the rabbit.²⁶,²⁷ HDL cholesterol is lowered in CETP-producing transgenic mice, and this effect appears to be dependent on the plasma level of CETP activity.²⁸,²⁹ Although not consistently observed,²⁴ an inverse relation between HDL cholesterol and CETP activity has also been found in humans.³⁰–³² The lack of a correlation between LCAT activity and HDL-CE in smokers, as opposed to the positive correlation in nonsmokers, would suggest that this enzyme-product relation is obscured by an enhanced CETP-mediated transfer of CE from HDL toward lipoproteins of lower density. Of note, the process of CE transfer in vitro is dependent on both the activity of CETP as such and the composition and concentration of CE donor and acceptor lipoproteins.³³–³⁶ For instance, elevations in VLDL-TG may enhance the net mass transfer of CE out of HDL.³¹ It is unlikely that this effect was operating in our study subjects because TG levels were similar in smokers and nonsmokers. PLTP is presumed to facilitate the transfer of phospholipids from TG-rich lipoproteins toward HDL during lipolysis.⁴ Since phospholipid-enriched HDL₃ appear to be better substrates for CETP, it is possible that CETP and PLTP act synergistically.⁶ As expected, a positive correlation of VLDL+LDL-CE with CETP activity was found,⁷,¹¹,¹⁰ but such a correlation with PLTP activity has not been demonstrated before. The extent to which these relations of lipid transfer protein activities to lipoprotein CE levels coincide with an altered CE mass transfer awaits further study.

Several mechanisms can be responsible for the higher lipid transfer activities in smokers. High levels of CETP activity are likely to be due to an increase in its mass concentration in plasma, since plasma CETP activity has been found to be closely correlated with CETP mass.⁶,¹⁰,²¹ Alternatively, a decrease in putative inhibitor activity could increase CETP activity.³² The interrelation between the activities of CETP and PLTP in smokers raises the possibility of a shared disturbance in the regulation of these proteins. In smokers the higher levels of VLDL+LDL-CE are thought to be caused partly by an increase in hepatic lipoprotein synthesis.³³ In addition, differences in dietary habits in smokers, such as higher intake of total fat³⁴ and lower intake of polyunsaturated fat,³⁵ may contribute to their higher levels of VLDL+LDL cholesterol. Diet-induced changes in plasma CETP are in part genetically determined and are well related to changes in VLDL+LDL cholesterol.³⁶,³⁷ As dietary habits were not analyzed in our study, the higher levels of CETP activity in smokers could be directly caused by an effect of smoking but also by associated changes in dietary factors.

In conclusion, lipid transfer proteins could play a role in causing or aggravating unfavorable lipoprotein changes. Even in otherwise normolipidemic men, there...
is an association between lower levels of HDL cholesterol, higher lipid transfer protein activities, and cigarette smoking.

Acknowledgments

We are indebted to M.M. Geelhoed-Mieras, T. van Gent, L.M. Scheek, and H. Breukelman for expert technical assistance. Dr W.J. Sluiter is acknowledged for statistical advice.

References

32. Nishide T, Tollefsen JH, Albers JJ. Inhibition of lipid transfer by a unique high density lipoprotein subclass containing an inhibitor protein. J Lipid Res. 1989;30:149-158.
Higher plasma lipid transfer protein activities and unfavorable lipoprotein changes in cigarette-smoking men.
R P Dullaart, K Hoogenberg, B D Dikkeschei and A van Tol

Arterioscler Thromb Vasc Biol. 1994;14:1581-1585
doi: 10.1161/01.ATV.14.10.1581
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1994 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/14/10/1581

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/