Smoking Prevents the Intravascular Remodeling of High-Density Lipoprotein Particles: Implications for Reverse Cholesterol Transport

Águeda C.M. Zaratin, Eder C.R. Quintão, Andrei C. Sposito, Valéria S. Nunes, Ana Maria Lottenberg, Richard E. Morton, and Eliana C. de Faria

Smoking is a leading cause of atherosclerosis acting trough a wide spectrum of mechanisms, notably the increase of the proatherogenic effect of dyslipidemia. However, a severe atherosclerotic disease is frequently observed in smokers who do not present an overt dyslipidemia. In the present study, we sought to determine if abnormalities in lipid metabolism occur in normolipidemic smokers, focusing especially on the components of intravascular remodeling of high-density lipoprotein (HDL) For this purpose, we measured lipid transfer proteins and enzymes involved in the reverse cholesterol transport (RCT) system in 29 adults: 15 smokers and 14 controls. The blood samples were drawn in the fasting state, immediately after the smokers smoked 1 cigarette. The composition of HDL particles was analyzed after isolation of HDL fractions by microultracentrifugation. We observed that normolipidemic smokers present higher total plasma and HDL phospholipids (PL) ($P < .05$), 30% lower postheparin hepatic lipase (HL) activity ($P < .01$), and 40% lower phospholipid transfer protein (PLTP) activity ($P < .01$), as compared with nonsmokers. The plasma cholesteryl ester transfer protein (CETP) mass was 17% higher in smokers as compared with controls ($P < .05$), but the endogenous CETP activity corrected for plasma triglycerides (TG) was in fact 57% lower in smokers than in controls ($P < .01$). Lipid transfer inhibitor protein activity was also similar in both groups. In conclusion, the habit of smoking induces a severe impairment of many steps of the RCT system even in the absence of overt dyslipidemia. Such an adverse effect might favor the atherogenicity of smoking.

ALTHOUGH MOST clinical attention has been directed to the cholesterol content of high-density lipoprotein (HDL) particles, HDL composition is an important indicator of HDL functionality. Indeed, a dynamic and continuous modeling of HDL is essential for the full functionality of these particles.[1] Metabolic steps, such as free-cholesteryl esterification by lecithin:cholesterol acyl transferase (LCAT), triglyceride (TG) hydrolysis by lipoprotein lipase (LPL), and interchange of phospholipid and cholesteryl ester between HDL and other lipoproteins by cholesteryl ester transfer protein (CETP), are globally implicated in the antiatherogenic, multistep process of reverse cholesterol transport (RCT).[2] In addition, hepatic lipoprotein lipase (HL) and the phospholipid transfer protein (PLTP) are involved in the interconversion of HDL subfractions, generating better acceptors for cell cholesterol efflux.[3] Thus, intravascular remodeling of HDL particles modulates their function and therefore their role in the prevention of cholesterol accumulation in the artery wall.

Cigarette smoking has been identified as an independent and strong risk factor for coronary heart disease (CHD).[4] The underlying mechanisms responsible for this association are complex and only partially understood. Regarding the lipoprotein metabolism, several proatherogenic modifications of plasma lipids and lipoproteins[5] have been described. The meta-analysis of 54 published studies by Craig et al[6] shows an increase in plasma concentrations of cholesterol (3%), TG (9.1%), very–low-density lipoprotein (VLDL)-cholesterol (10.4%), and low-density lipoprotein (LDL)-cholesterol (1.7%) and a reduction in the concentrations of HDL-cholesterol (5.7%) and apolipoprotein (apo) AI (4.2%), in smokers as compared with nonsmokers. This greater impact on HDL-cholesterol and apo AI levels suggests that abnormalities in HDL metabolism could constitute a major metabolic effect of the smoking habit. Thus, even in normolipidemic subjects, smoking may possibly confer atherogenicity by preventing the intravascular remodeling of HDL and, as a consequence, by disturbing the RCT. Aiming to test this hypothesis, we investigated the activities of LPL, HL, LCAT, CETP, PLTP, and the lipid transfer inhibitor protein (LTIP or apo F) in a homogenous group of normolipidemic men who displayed a moderate to chronic use of cigarettes.

MATERIALS AND METHODS

Experimental Protocol

Twenty-nine normolipidemic male volunteers, 15 smokers and 14 controls, were selected from 90 subjects screened to participate in the protocol. All procedures followed were in accordance with the Ethics Committee of the School of Medicine of the University of Campinas. All participants were selected for their normolipidemic profiles (cholesterol < 200 mg/dL, TG < 150 mg/dL, and LDL-cholesterol < 100 mg/dL) in accordance with the standards of the National Cholesterol Education Program (NCEP III), with body mass index (BMI) ≤ 25 kg/m^2. The intake of fat, carbohydrates, and protein in both groups was roughly estimated by a diet questionnaire as being 25% to 30% lipids, 50% to 60% carbohydrates, and 10% to 15% proteins. Enrolled subjects were selected so as to be comparable on the basis of age and BMI. Smokers had smoked at least 10 cigarettes/day for at least 1 year and the controls never smoked. Participants had their blood pressure and

From the Department of Clinical Pathology and Center for Experimental Medicine and Surgery, Faculty of Medical Sciences, University of Campinas, Campinas; Lipid Laboratory, University of São Paulo Medical School, São Paulo; Heart Institute (InCor), Zerbini Foundation, Brasilia, Brazil; and the Department of Cell Biology, Lerner Research Institute, Cleveland Clinic Foundation, Cleveland, OH.

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Address reprint requests to Eliana Cotta de Faria, MD, PhD, Department of Clinical Pathology, Faculty of Medical Sciences, University of Campinas, Caixa Postal 6111, CEP 13083-970, Campinas SP, Brazil.

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anthropometric data measured during the clinical exam and answered a questionnaire on the presence of CHD risk factors, diet, alcohol intake, and physical exercise. Smokers did not refrain from smoking before the protocol started. Fasting blood samples were drawn after a 10-minute rest from all participants and 5 minutes after the smokers had smoked 1 cigarette to maximize the effects of smoking and to allow for an identical time-dependent effect of smoking among the participants. New blood samples were then collected 10 minutes after heparin injection (Liquemine, 100 UI/kg of body weight, Roche, Basel, Switzerland) for the measurement of the LPL activity.

Biochemical Measurements

Cotinine was analyzed by gas liquid chromatography[7] by Dr Colin Feyerabend at the Medical Toxicology Unit, New Cross Hospital, London, England.

Plasma glucose was assayed in an automated system (Selectra, Merck, Mannheim, Germany) using an enzymatic-colorimetric method by Labtest (Belo Horizonte, MG, Belo Horizonte, Brazil). Fibrinogen was determined by the method of Clauss and insulin by radioimmunoassay from Linco (São Paulo, SP, Brazil).

Lipid, Apolipoprotein, and Lipoprotein Analysis

Total cholesterol and TG were determined using enzymatic diagnostic reagents provided by Labtest (Belo Horizonte, MG, Brazil) in an automated system (Selectra, Merck). Free fatty acids (FFA), unesterified cholesterol (UC), and phospholipids (PL) were measured by enzymatic colorimetric assays (Waco Bioproducts, Belo Horizonte); cholesteryl ester (CE) was defined as the difference between total cholesterol and UC.

HDL-cholesterol was measured in the supernatant after precipitation of apoB-containing lipoproteins with dextran sulfate and $MgCl_2$. LDL-cholesterol was estimated by Friedewald's formula. HDL_2 and HDL_3 were separated by sequential microultracentrifugation (Airfuge, Beckman, Palo Alto, CA).[8] Apolipoproteins AI, B100, and lipoprotein [Lp](a) were measured by nephelometric assays in the system Array 360 (Beckman).

LCAT, Lipases, Transfer Proteins, and LTIP Analysis

Lecithin:cholesterol acyl transferase activity was assayed by an endogenous radiometric method using HDL as the substrate and source of LCAT (15). HDL was incubated with [1,2-^{3}H(N)]cholesterol for 24 hours. [^{3}H]cholesterol-labeled HDL was incubated at 37°C for 30 minutes and the free and esterified fractions of cholesterol were then separated by thin layer chromatography. LCAT activity was expressed as the cholesteryl esterification rate as percent/30 minutes.

The CETP activity was measured by an endogenous assay.[9] Aliquots of the whole plasma (in which LCAT activity was inhibited by dithionitrobenzoic acid [DTNB] 9 μL/mL) were added to HDL-[^{3}H]cholesteryl ester fractions and simultaneously incubated at 4°C and 37°C for 4 hours. Apo B–containing lipoproteins, present in the incubation mixture, were then precipitated; the CE radioactivity in the supernatant represented the net rate at which CE mass was transferred, and the values expressed as a percent of [^{3}H] cholesteryl ester transferred every 4 hours depended upon the plasma concentrations of HDL, TG-rich lipoproteins, and CETP simultaneously.

CETP concentration was measured by radioimmunoassay by Dr. Laurent Lagrost[10] of the Laboratoire de Biochimie des Lipoprotéines, Hôpital du Bocage, Dijon, France.

LTIP activity was measured as previously described.[11] This radiometric method uses exogenous CETP, ^{3}H-CE LDL, and unlabeled HDL incubated in the presence of lipoprotein-deficient plasma as the LTIP source. LTIP activity was determined by comparing CETP activity in the presence and absence of the added LTIP source. To minimize interassay variability, LTIP activity values (% inhibition/mL) were normalized to the LTIP activity measured in a lipoprotein-deficient plasma standard run in the same experiment. These normalized values are reported as relative inhibition/mL.

The PLTP was measured by an exogenous radiometric method using phospholipid lipossomes as the substrate[12] and an HDL pool obtained from plasma donors as the acceptor. The activity was expressed as the rate of radioactively labeled phospholipid transfer/hour.

LPL and HL activities were quantified in postheparin plasma samples (10 minutes after the intravenous injection of heparin, 100 U kg^{-1} body weight), on the basis of fatty acid release, using a radiolabeled triolein emulsion as the substrate and NaCl (1 mol/L) as the LPL inhibitor[13]; the results were expressed as nanomoles FFA/mL/h.

The assays for CETP concentration, LTIP, PLTP, and LPL were conducted in triplicate. The interassays coefficients of variation were 16%, 12%, 2%, 9%, and 8%, respectively, for CETP, LTIP, PLTP, LPL, and HL.

Statistics

Differences between the groups were measured by the Mann-Whitney test. The Spearman test was used to correlate the variables. Values were considered to be significant at $P < .05$.

RESULTS

Clinical and Biochemical Characteristics of the Participants

Table 1 summarizes the clinical and biochemical characteristics of the participants. Both groups were very similar except for their diastolic blood pressure, significantly lower in smokers. All individuals were healthy young male adults and did not present centripetal fat distribution. No group differences were found in metabolic variables, such as glucose and insulin, and these values were within the range of reference values for healthy adults. No clinical signs of atherosclerosis were found in either group.

Smokers smoked an average of 19 cigarettes a day. They presented plasma cotinine levels above 184 ng/mL, defined as the range for heavy smokers.[14] The plasma fibrinogen was significantly higher in smokers.

Lipids, Apolipoproteins, and Lipoproteins

As shown in Table 2, the individuals studied had plasma levels within the reference limits. Concentrations of cholesterol, LDL-cholesterol, HDL-cholesterol, apo B-100, apo AI, FFA, and Lp(a) did not differ between smokers and controls. TG and PL plasma concentrations were 30% and 14% higher, respectively, in smokers ($P < .05$). When the lipid composition of HDL subfractions was analyzed, there were no differences between the groups, with the exception of a 21% increase in HDL_3-PL in smokers ($P < .05$), and this finding could account for their higher plasma PL.

LCAT, Lipases, and Transfer Proteins

Table 3 summarizes the values for the activities of LCAT, lipases, and transfer proteins. LCAT activity in smokers did not differ from that of controls. Fasting postheparin plasma LPL was similar in both groups, but the activity of HL was 30% lower in smokers. PLTP activity was reduced in smokers by 30%. When corrected for PL as the ratio PLTP/PL, the difference was even larger (40%).

Table 1. Anthropometric and Biochemical Characteristics of Smokers and Controls

	Smokers (n)	Controls (n)
Age (yr)	28 ± 1 (15)	26 ± 1 (14)
	19-34	19-35
Height	1.8 ± 0.02 (15)	1.8 ± 0.02 (14)
	1.6-1.9	1.7-1.9
Weight	70 ± 2.6 (15)	69 ± 2.1 (14)
	52-83	55-85
BMI (kg/m^2)	22 ± 0.5 (15)	22 ± 0.6 (14)
	19-25	19-25
Waist/hip	0.8 ± 0.01 (13)	0.8 ± 0.02 (14)
	0.8-0.9	0.7-1.0
Blood pressure (mm Hg)		
Systolic	122 ± 5 (13)	121 ± 3 (14)
	100-180	100-140
Diastolic	76 ± 2 (13)*	81 ± 1 (14)
	70-90	70-90
No. of cigarettes/d	19 ± 1.0 (15)	—
	10-20	
Smoking time (yr)	11 ± 1 (15)	—
	4-18	
Cotinine (ng/mL)	261 ± 28 (14)	—
	141-492	
Glucose (mg/dL)	88 ± 3 (14)	84 ± 2 (14)
	74-119	72-94
Insulin (μIU/mL)	9 ± 1 (15)	7 ± 1 (14)
	5-19	4-12
Fibrinogen (mg/dL)	307 ± 19 (15)†	233 ± 13 (12)
	220-430	140-324

NOTE. Data presented as means ± SEM and ranges (n = number of subjects).

Abbreviation: BMI, body mass index.

Statistical comparisons between smokers and controls by Mann-Whitney test: *$P < .05$; †$P < .01$.

The CETP mass was 17% higher in smokers, but the endogenous CETP activity was not statistically different. After correction for the higher TG level found in smokers, as the CETP activity/TG ratio, the endogenous CETP activity was in fact 57% lower in smokers. The CETP specific activity corrected by TG (% of CE transferred per hour per milligram of CETP per TG) was lower in smokers ($P = .03$). LTIP activity was similar in the groups. In smokers, plasma total cholesterol ($r = 0.66$) and LDL-cholesterol ($r = 0.58$) concentrations correlated positively with levels of cotinine, the main catabolic product of nicotine.

DISCUSSION

In the present study, we sought to determine if abnormalities occur in the lipid metabolism of normolipidemic smokers, placing special emphasis on the components of intravascular remodeling of HDL. Even though selected as normolipidemic, the smokers presented increases in blood PL and in the HDL_3-PL subfraction and reductions in the activities of HL, PLTP, and CETP. Because a large body of evidence supports the antiatherosclerotic role of RCT, such alteration of HDL phenotype and reduction of activity in multiple steps of RCT indicates that smoking may induce proatherogenic changes in lipid metabolism even in the absence of overt dyslipidemia.

Normolipidemic smokers presented significantly higher plasma and HDL phospholipid levels (+14%) as compared with nonsmokers. The augmentation in phospholipid content of HDL was observed in all HDL fractions, but was clearly more pronounced in the smaller particles HDL_3 (+25%). Such modification in HDL phenotype has been associated with an increased atherogenic risk[15] and could be secondary to changes in the activity of PLTP, which is a plasma glycoprotein that promotes the transfer of PL between HDL and other plasma lipoproteins.[16] In line with previous reports,[17] we observed that smokers present a significant decrease in PLTP activity even after correction by PL concentration, favoring the accumulation of PL on the HDL surface. Moreover, the decrease in HL or CETP activities observed in these subjects may also favor the increase of the phospholipid content of HDL. A reduced activity of HL increases the amount of core TG and, consequently, the size of HDL particles. Such enlargement in HDL size results in a retention of additional surface components (apoA-I, PL, unesterified cholesterol) that are required to accommodate

Table 2. Lipids, Lipoproteins, and Apolipoproteins and Composition of HDL as Total and Unesterified Cholesterol, Cholesteryl Ester, Phospholipids, and Triglycerides in Smokers and Controls

	Smokers	Controls
Cholesterol (mg/dL)	157 ± 6 (15)	156 ± 6 (14)
	132-199	108-196
LDL-cholesterol (mg/dL)	89 ± 6 (15)	96 ± 6 (14)
	61-128	59-122
HDL-cholesterol (mg/dL)	49 ± 3 (15)	45 ± 3 (14)
	34-77	32-71
Triglyceride (mg/dL)	99 ± 11 (15)*	69 ± 12 (14)
	44-161	35-203
Free fatty acids (mEq/L)	0.6 ± 0.09 (14)	0.8 ± 0.09 (14)
	0.2-1,2	0.2-1.5
Phospholipids (mg/dL)	140 ± 6 (14)*	120 ± 8 (14)
	104-180	91-209
Apolipoprotein A-I (mg/dL)	134 ± 7 (15)	125 ± 5 (14)
	102-189	102-165
Apolipoprotein-B (mg/dL)	64 ± 3 (15)	67 ± 4 (14)
	42-87	41-94
Lp(a) (mg/dL)	20 ± 5 (15)	11 ± 3 (13)
	2-49	3-37
HDL_2-free-cholesterol (mg/dL)	5 ± 1 (14)	4 ± 1 (14)
	2-12	2-8
HDL_2-cholesteryl-ester (mg/dL)	10 ± 2 (14)	11 ± 1 (14)
	3-21	4-19
HDL_2-TG (mg/dL)	15 ± 2 (15)	12 ± 2 (14)
	7-39	4-20
HDL_2-PL (mg/dL)	23 ± 2 (14)	20 ± 3 (14)
	13-39	2-53
HDL_3-free-cholesterol (mg/dL)	12 ± 2 (14)	11 ± 1 (14)
	6-27	5-19
HDL_3-cholesteryl-ester (mg/dL)	29 ± 3 (14)	25 ± 3 (14)
	10-50	6-53
HDL_3-TG (mg/dL)	28 ± 3 (15)	24 ± 2 (14)
	18-58	12-41
HDL_3-PL (mg/dL)	74 ± 4 (14)*	59 ± 6 (14)
	55-96	12-104

NOTE. Data presented as means ± SEM and ranges (n = number of subjects).

Statistical comparisons between smokers and controls by Mann-Whitney test: *$P < .05$.

Table 3. Esterification Rate of Free Cholesterol, Lipoprotein Lipase, Hepatic Lipase, and Phospholipid Transfer Protein Activities and Percentage of Transfer of Cholesteryl Ester to Apo B–Containing Lipoproteins, CETP Mass, CETP Specific Activity, and Lipoprotein Transfer Inhibitor Protein Activity in Smokers and Controls

	Smokers (n)	Controls (n)
LCAT (%/30 min)	6.9 ± 0.6 (13)	7.0 ± 0.7 (14)
	3.1-10.0	3.8-11.2
LPL (nmol FFA/mL/h)	3,094 ± 384 (15)	2,819 ± 299 (14)
	1,208-6,189	950-5,773
HL (nmol FFA/mL/h)	3,384 ± 359 (15)*	4,699 ± 298 (14)
	1,157-6,169	2,391-6,824
PLTP (%/h)	11.8 ± 1.0 (14)*	16.8 ± 0.5 (14)
	4.5-15.9	13.9-21.6
PLTP/PL (%/mg/dL)	0.09 ± 0.01 (14)*	0.15 ± 0.01 (14)
	0.04-0.13	0.08-0.24
CETP (%)	31 ± 6 (10)	39 ± 4 (10)
	8-55	22-64
CETP/TG (%/mg/dL)	0.3 ± 0.03 (10)*	0.7 ± 0.2 (10)
	0.07-0.4	0.1-1.8
CETP (mg/L)	2.9 ± 0.1 (13)†	2.4 ± 0.1 (11)
	2.6-3.2	2.1-2.7
CETP specific activity (% CE/h/mg CETP)	12 ± 2 (8)	15 ± 2 (8)
	6-18	9-21
LTIP (rel · inh/mL)	498 ± 68 (14)	599 ± 79 (14)
	244-752	221-1,213

NOTE. Data presented as means ± SEM and ranges (n = number of subjects).
Statistical comparisons between smokers and controls by Mann-Whitney test: *$P \leq .01$; †$P \leq .05$.

the increased amount of core lipids. Similarly, when CETP activity is reduced, the transfer of LCAT-derived cholesteryl esters from HDL to other lipoprotein fractions is reduced and, thus, the total core lipid content and the particle surface of HDL increase. In accordance with the study of Mero et al,[18] despite the higher CETP mass found in smokers, we observed a reduced net transfer of endogenous cholesteryl esters. Therefore, a reduced activity of PLTP, HL, and CETP might all have accounted for the increase in HDL-PL observed in normolipidemic smokers. We postulate that hyperlipidemic smokers would present even lower activities of the above proteins.

The mechanism(s) underlying the reduction in the CETP activity in smokers is unknown, but would be partially related to the inhibitory effect of LTIP on CETP activity or to the reduction in HL activity. LTIP or apo F regulates the interaction of CETP with lipoproteins and is postulated to enhance the ability of CETP to stimulate RCT.[19] However, we measured LTIP activity in smokers for the first time and did not find significant differences as compared with nonsmokers. On the other hand, the reduced HL activity observed in smokers may favor the reduction in CETP activity; it has been shown that HDL-TG hydrolysis by HL facilitates CETP-mediated transfer of TG from TG-rich lipoproteins to HDL in exchange for cholesteryl esters. Thus, it is likely that the reduction in the net transfer of cholesteryl ester in smokers results from chemical modifications of the donor and acceptor lipoprotein pools.

Although smoking did not induce hypercholesterolemia, there was a strong positive association between the levels of serum cotinine and LDL-cholesterol, suggesting that cigarette smoking may possibly increase the residence time of LDL particles in the circulation. Consistent with this assumption, it has been shown that LDL particles from smokers have higher anodic electrophoretic mobility and greater crosslinking of their apoprotein by nondisulfide bonds; thus in this way, they show a reduced binding affinity with liver membrane receptors.[20] Besides the typically augmented production of reactive oxygen species in smokers, such an extension of LDL residence time increases the substrate available for generation of oxidized LDL, thereby inducing inflammatory stimulus and plaque formation in the artery wall. In fact, data from our laboratory indicated that smokers have higher levels of oxidized LDL than nonsmokers.[21] Although normotriglyceridemic, smokers also had TG levels 30% higher than controls, a finding that could reflect a decreased lipolysis rate of TG-rich lipoproteins in these individuals. Consistent with this assumption, we observed a 25% lower serum level of FFA in smokers as compared with controls. LPL activity was similar in the 2 groups, in accordance with the majority of the studies in the literature. However, we found a significant reduction in HL activity in smokers, which might reduce the residual lipolysis of TG-rich lipoprotein remnants[22] and, consequently, increase plasma TG levels[23] in those subjects and also increase the residence time of HDL.[24] In agreement with previous studies, we did not observe significant difference in LCAT activity between the groups.

In conclusion, taken together, the results of this study show that the smoking habit induces impairment in many steps of the RCT system even in the absence of overt dyslipidemia. Such effect might favor the atherogenicity of smoking.

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