Modification of LCAT Activity and HDL Structure
New Links Between Cigarette Smoke and Coronary Heart Disease Risk

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Abstract The mechanism(s) through which smoking influences the progression of atherosclerosis is poorly understood. Recent evidence suggests that oxidants present in the gas phase of cigarette smoke are involved. We exposed human plasma to the filtered gas phase of cigarette smoke to assess its effects on plasma components involved in the antiatherogenic reverse cholesterol transport pathway. In our model, freshly isolated plasma (24 mL) was exposed to filtered air or gas-phase cigarette smoke for up to 6 hours at 37°C. Lecithin-cholesterol acyltransferase (LCAT) activity was dramatically inhibited by cigarette smoke. A single 15-minute exposure to the smoke from an eighth of a cigarette was sufficient to reduce LCAT activity by 7%; additional exposures resulted in further decreases in activity. At 6 hours, only 22% of control LCAT activity remained in plasma exposed to smoke. Compared with control, gas-phase cigarette smoke–exposed plasma possessed high-density lipoprotein (HDL) with increased (16%) negative charge and with cross-linked apolipoproteins AI and AII. These data demonstrate that gas-phase cigarette smoke can inhibit a key enzyme (LCAT) and modify an integral lipid transport particle (HDL) that are essential components for the normal function of the reverse cholesterol transport pathway. Gas-phase cigarette smoke–induced modification of the reverse cholesterol transport pathway may provide a new mechanistic link between cigarette smoke and coronary heart disease risk. (Arterioscler Thromb. 1994;14:248-253.)

Key Words • lecithin-cholesterol acyltransferase • reverse cholesterol transport • HDL • cigarette smoke • coronary heart disease

Epidemiological studies have established that plasma high-density lipoprotein (HDL) concentrations are inversely correlated with coronary heart disease risk.1-2 The mechanism for a possible direct protective effect of HDL is unknown, although it has been suggested that HDL participates in a cholesterol transport pathway that removes excess cholesterol from macrophage-derived foam cells in early atherosclerotic lesions and transports it back to the liver for catabolism (for reviews see References 3 through 5). A growing body of evidence supports this putative "reverse cholesterol transport pathway," although the physiochemical properties of HDL particles accepting cholesterol from foam cells and the signaling mechanism responsible for releasing stored intracellular cholesterol ester from foam cells are still somewhat controversial. The major steps of the reverse cholesterol transport pathway are as follows: net efflux of cholesterol from cholesterol-laden cells to HDL; esterification of cholesterol on HDL mediated by lecithin-cholesterol acyltransferase (LCAT); transfer of esterified cholesterol from HDL to lipoproteins of lower density by cholesteryl ester transfer protein; and clearance of lipoprotein-associated cholesterol by the liver. The plasma enzyme LCAT is an essential component of this pathway because it maintains the unesterified cholesterol concentration gradient, which permits cholesterol efflux and net transport. LCAT requires apolipoprotein (apo) AI, the major protein component of HDL, as a cofactor to express optimal activity.6

Cigarette smoking is associated with premature atherosclerosis and increased mortality from coronary heart disease.7-9 The pathophysiological mechanisms that underlie the influence of smoking on coronary heart disease are not well defined, although evidence suggests that the mechanism involves altered lipoprotein metabolism. Epidemiological studies have shown that plasma HDL concentrations are reduced in cigarette smokers.8-10 It has also been shown that acute cigarette smoke exposure in humans results in subtly modified low-density lipoproteins (LDLs) with enhanced atherogenic potential.11 The mechanism by which cigarette smoke affects plasma LDL and HDL is not known, although it is possible that oxidative damage to protein and/or lipid constituents is involved.

The gas phase of cigarette smoke is chemically complex, possessing a variety of components (eg, free radicals12-13 and aldehydes14-15) that can potentially damage proteins and lipids in vitro, as suggested by the study of Harats and coworkers.11 There are two potential mechanisms by which reactive smoke components can produce their deleterious effects on essential plasma constituents: (1) Indirectly, gas-phase cigarette
smoke may activate macrophages and/or neutrophils in
the lung, which in turn may release enzymes and oxidants (eg, myeloperoxidase, elastase, and free radicals) capable of damaging plasma lipids and proteins. (2) Directly, since the lung possesses an extremely large surface area for gas exchange, it is possible that gas-phase cigarette smoke components interact with plasma constituents, including HDL, LDL, and LCAT, in an in vitro model system. We were particularly interested in investigating the susceptibility of HDL and LCAT to cigarette smoke modification because of epidemiological reports indicating reduced HDL cholesterol in smokers and because modification of one or both of these plasma components may decrease the efficient removal of cholesterol from cells. The latter can increase the risk for cardiovascular disease.

Methods

Subjects

Blood was obtained from fasted healthy adult volunteers who had given informed consent. Volunteers were nonsmokers who did not supplement their diets with vitamins. Heparin was used to prevent coagulation, and plasma was separated from cellular blood components by low-speed centrifugation (2000g, 4°C). Gentamicin sulfate was added to plasma to prevent microbial contamination. Experiments were performed on the same day that blood was drawn. Plasma concentrations for total cholesterol, HDL cholesterol, and total triacylglycerol expressed as the mean±SD for eight volunteers (five men and three women) were 210±41, 56±20, and 152±112 mg/dL, respectively.

Plasma Incubations With Gas-Phase Cigarette Smoke

To expose freshly isolated human plasma to gas-phase cigarette smoke, a smoking apparatus similar to that described by Frei et al was used. Negative pressure was introduced with a house vacuum into a 250-mL side-arm flask containing 24 mL plasma. The negative pressure was subsequently used to draw smoke from a lit Kentucky 2R1 research cigarette through a Cambridge filter (rated to exclude particles >0.01 μm) into the flask. The flask was then sealed and incubated in a shaking 37°C water bath in the dark. At 15-minute intervals, additional filtered smoke was introduced into the flask. The filtered gas phase from an eighth of a cigarette was used for each exposure (one-puff exposure); the equivalent of one cigarette was used every 2 hours. Control flasks were handled identically to experimental flasks except that filtered air was used instead of cigarette smoke. Changes in plasma LCAT activity, plasma antioxidant concentrations, and physicochemical properties of lipoproteins were examined at hourly intervals for 6 hours.

To investigate whether alteration of free cysteine residues in LCAT by cigarette smoke may contribute to inhibition of LCAT activity, we added reduced glutathione (GSH) to smoke-exposed plasma in three separate experiments. Incubations were performed as described above, except that two flasks were exposed to gas-phase cigarette smoke; GSH was added to one of the two smoke-exposed flasks before each smoke exposure. Preliminary experiments indicated that although concentrations of GSH >0.5 mmol/L had more pronounced protective effects, a precipitate formed before the 6-hour time point. We therefore used 0.5 mmol/L GSH in these experiments. LCAT activity was assessed at 1, 3, and 6 hours.

Plasma LCAT Activity

LCAT activity was assessed by the exogenous "common substrate" (ie, proteoliposome) method of Albers et al. This assay has been shown to be dependent on the amount of active enzyme and largely independent of the substrates and cofactors in the plasma sample.

Plasma Antioxidant Concentrations

Because of the liability of ascorbate, plasma samples to be assayed for this vitamin were extracted immediately after incubation and stored in liquid nitrogen until assayed. Ascorbate and urate concentrations were determined by the method of Kutnik et al. α-Tocopherol concentrations were measured by high-performance liquid chromatography.

Characterization of Lipoproteins

Plasma samples were subjected to agarose gel electrophoresis to determine whether electrophoretic mobilities of specific lipoprotein classes were altered by smoke exposure. Beckman Paragon Lipo gels were used according to the manufacturer's recommendations. Mobility was quantified by measuring the distance from the application point of the samples to the trailing edge of the stained lipoprotein band. Changes in mobility are expressed as the percent increase in smoke-exposed samples compared with the appropriate air control.

LDL and HDL were isolated from plasma by sequential ultracentrifugation using standard techniques. Isolated fractions were subsequently dialyzed to a lower-salt background (150 mmol/L NaCl, 1 mmol/L EDTA; pH 7.4) and assayed for protein. Cigarette smoke-induced peroxidation of HDL and LDL was estimated by the thiobarbituric acid–reactive substances (TBARS) assay. Total fatty acid composition of HDL isolated from samples exposed to 6 hours of air and 6 hours of smoke was determined in three subjects by gas-liquid chromatography. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) of HDL and LDL apolipoproteins was performed under nonreducing conditions as described by Laemmli using 4% to 20% gels. Western blot analyses of SDS gels were carried out with polyclonal antibodies monospecific for apo AI and AII.

Results

Effect of Cigarette Smoke on LCAT Activity

In vitro exposure of plasma to filtered cigarette smoke dramatically inhibited LCAT activity (Fig 1). At 1 hour (four puff equivalents), the smoke-exposed plasma possessed less than half the activity (44%) of the air-exposed control. Longer smoke exposure resulted in further decreases in activity. At 6 hours, only 22% of control activity was observed in the smoke-exposed plasma. In a separate set of experiments, changes in LCAT activity as a function of smoke exposure were evaluated at early time points, including 15, 30, and 45 minutes (ie, exposures of 1, 2, and 3 puffs, respectively). A single 15-minute exposure was sufficient to produce a decrease (n=4) in LCAT activity; longer exposures of 30 and 45 minutes resulted in significant decreases in LCAT activity of 15±10% and 21±12%, respectively. The largest single decrement in LCAT activity was observed after the fourth smoke exposure (ie, 1 hour), suggesting that plasma has a limited capacity to protect LCAT from filtered cigarette smoke–induced inhibition. Inhibition could not be reversed by dialysis of the sample after exposure to cigarette smoke, nor was it reversed upon addition of β-mercaptoethanol (5 mmol/L), the reducing agent included in our LCAT assay system.
FIG 1. Graph showing plasma lecithin-cholesterol acyltransferase (LCAT) activity: effect of time of exposure to the gas phase of cigarette smoke. Incubations were performed with 24 mL fresh plasma in side-arm filter flasks (250-mL flasks) at 37°C (in the dark), essentially as described by Frei et al. The gas phase of cigarette smoke (University of Kentucky 2R1 research cigarettes of known tar and nicotine content) was introduced at regular intervals into the flask through a Cambridge filter. Aliquots of plasma were removed from control (exposed to filtered air) and experimental (exposed to the gas phase of cigarette smoke) flasks at various times, depending on the experiment, between 1 and 6 hours of incubation. Data are expressed as the mean±SD for three separate experiments. No significant changes in LCAT activity were observed in control samples exposed to filtered air. 100% LCAT activity=107±13 nmol cholesteryl ester formed per hour per milliliter plasma.

Effect of Cigarette Smoke on Endogenous Antioxidant Concentrations

Plasma ascorbate, urate, and α-tocopherol concentrations declined at different rates during exposure to the gas phase of cigarette smoke (Fig 2). Ascorbic acid rapidly disappeared and was not detectable after 1 hour of exposure (the earliest time point we examined). This observation is consistent with that of Frei et al., in which ascorbate concentrations declined to zero within 0.5 hour of exposure of plasma to filtered cigarette smoke. α-Tocopherol and urate concentrations in our studies decreased gradually after an initial lag period of approximately 1 hour to 40% and 25%, respectively, of control values at 6 hours (Fig 2). Therefore, depletion of plasma antioxidant concentrations (Fig 2) induced by gas-phase cigarette smoke does not appear to parallel smoke-induced decreases in LCAT activity (Fig 1).

Effect of GSH on Cigarette Smoke–Induced LCAT Inhibition

Addition of GSH to plasma before exposure to gas-phase cigarette smoke resulted in significant protection of LCAT activity (Fig 3). After 3 hours of smoke exposure, the GSH-supplemented plasma retained 65±4% of control LCAT activity compared with 42±7% for unsupplemented plasma. Significant protection of LCAT activity by GSH was also observed at 1 and 6 hours. These data suggest that thiol chemistry may be involved in the smoke-induced inhibition of LCAT.

Changes in electrophoretic mobility of the major lipoprotein classes after exposure of plasma to filtered cigarette smoke compared with air exposure were assessed by agarose gel electrophoresis (Fig 4). Each major lipoprotein class, after an initial lag period (>1 hour), displayed increased electrophoretic mobility (ie, lipoproteins were more negatively charged), and these changes tended to increase with time (Fig 4). LDL and HDL exhibited more rapid and pronounced increases in mobility than very-low-density lipoproteins (VLDLs). In general, the maximum mobility change observed in LDL exceeded that observed in HDL, which in turn exceeded that observed in VLDL. Changes in LDL mobility induced by gas-phase cigarette smoke were not accompanied by large increases in TBARS or in changes in apo B relative molecular weight. LDL TBARS expressed as malondialdehyde equivalents ranged from 1.5 to 2.9 nmol/mg protein for 4°C control and from 2.7 to 5.2 nmol/mg protein for 6-hour smoke exposures. SDS-PAGE of the isolated smoke-exposed LDL revealed no cross-linking or fragmentation of apo B (data not shown). These observa-

Fig 2. Graph showing plasma antioxidant concentrations (α-tocopherol, α; ascorbate, Δ; urate, O): effect of time of exposure to the gas phase of cigarette smoke. Incubations were performed as described in Fig 1. Data are expressed as the mean±SD for three separate experiments. Antioxidant data are given as percentages of their mean initial concentrations, which were as follows: ascorbate, 45±16 μmol/L; α-tocopherol, 17±9 μmol/L; urate, 213±36 μmol/L.

Fig 3. Bar graph showing plasma lecithin-cholesterol acyltransferase (LCAT) activity: effect of reduced glutathione (GSH) on cigarette smoke–induced inhibition of LCAT (GSH supplemented plasma, open bars; unsupplemented plasma, closed bars). Incubations were performed as described in Fig 1, except that two flasks were exposed to gas-phase cigarette smoke. GSH (0.5 mmol/L) was added to one of the two flasks before each smoke exposure; LCAT activity was determined at 1, 3, and 6 hours. Data are expressed as the mean±SD for three separate experiments.
Cross-linking of apo Al and apo All was observed at 1 hour (data not shown). The subtle alteration of LDL structure is also consistent with the observation of Harats et al.11 LDLs isolated from smokers are subtly altered so that they are more susceptible to oxidation.

Unlike LDL, the major protein components of HDL, apo Al and apo All, were rapidly altered by exposure of plasma to filtered cigarette smoke (Fig 5). SDS-PAGE of HDL isolated from smoke-exposed plasma revealed high-molecular-weight, cross-linked forms of these apo-lipoproteins (Fig 5). This cross-linking was observed with as little as 1 hour of smoke exposure (data not shown), suggesting that HDL apolipoproteins are relatively sensitive to the effects of gas-phase cigarette smoke. Western blot analysis demonstrated that the cross-linked HDL proteins were multimers containing apo Al, apo All, or apo Al plus apo All.

HDL lipid peroxidation assessed as malondialdehyde equivalents by the TBARS assay was uniformly low and not different between air- and smoke-exposed plasma (data not shown). Concentrations ranged from 0.8 to 1.4 nmol/mg protein, with higher concentrations generally associated with the 6-hour smoke exposure. Measurement of total HDL fatty acids in three separate experiments tended to confirm the TBARS data. No detectable differences in the average number of double bonds, the ratio of unsaturated to saturated fatty acids, or the ratio of arachidonic acid to palmitic acid could be detected between samples exposed to 6 hours of air and 6 hours of smoke (data not shown). These data suggest that lipid peroxidation may not play an important role in the observed HDL protein modifications.

**Discussion**

A novel finding of the present study is that in our in vitro model system, LCAT is extremely sensitive to inhibition by the gas phase of cigarette smoke. Exposures that produce a 56% decrease in LCAT activity in our model system (ie, four puffs) are roughly equivalent to exposing the plasma volume of an average man to the smoke of approximately two packs of cigarettes. It is unlikely that transfer of gas-phase cigarette smoke components into the interstitial fluid of the lung is anywhere near as efficient as that observed in our model system. It should be noted, however, that the amounts of smoke required for inhibition of LCAT activity are not large. Since LCAT is thought to play an essential role in efflux and net transport of cholesterol from cells, our data provide suggestive evidence that increased coronary heart disease risk in smokers may, in part, be related to alterations in the activity of this enzyme.

Smokers have decreased concentrations of HDL and are clearly at greater risk than nonsmokers for developing coronary heart disease.9 Interestingly, a cross-sectional study has demonstrated that smokers have reduced plasma LCAT concentrations (approximately 14%) compared with nonsmokers.10 We are unaware of any in vivo studies examining the acute effects of cigarette smoke on LCAT activity; studies in this area appear to be warranted.

LCAT is a highly glycosylated (20% by weight) protein of approximately 66 kD that possesses two disulfide bridges and two free cysteines.6,8 The reaction catalyzed by LCAT is the transfer of an acyl chain from the sn-2 position of phosphatidylcholine to the 3-hydroxyl group of cholesterol, forming lysophosphatidylcholine and cholesteryl ester, respectively. This reaction is thought to be mediated by a "catalytic triad" of Ser-His-Asp. According to several laboratories, Ser 218 is the reactive Ser residue,26,29 this residue is located in a pocket bounded by the two free cysteine residues of LCAT. It has been reported that modifying these free cysteines results in the loss of the catalytic activity of LCAT.30 In the present study, inhibition of LCAT...
activity by cigarette smoke was partially prevented by providing relatively large amounts of exogenous reduced GSH. This protective effect of GSH suggests that inhibition of LCAT by gas-phase cigarette smoke may involve modification of one or more of the free cysteines of LCAT.

In addition to inactivating LCAT, gas-phase cigarette smoke also modified HDL protein structure as reflected by cross-linking of apo AI and AII. Cross-linking was observed with as little as 1 hour of smoke exposure. Structural modifications of HDL proteins, particularly apo AI, can adversely affect the ability of apo AI to activate LCAT and thus impair the process of reverse cholesterol transport. Cross-linking of HDL proteins can also potentially impair the ability of HDL to promote movement of cholesterol from cell membranes (ie, impair the first step in the reverse cholesterol transport pathway, efflux of cholesterol from cells). In support of the latter contention, it has recently been demonstrated that HDL possessing copper-induced cross-linked apolipoproteins we observed in our concentrations observed in smokers are the result of inactivation of LCAT.

It has been shown that HDLc cross-linked with suberimidate has an increased rate of catabolism. This observation is of interest because a recent study examining the in vivo plasma compartment kinetics of apo AI in smokers versus nonsmokers showed that the residence time for apo AI in smokers was reduced by approximately 30% compared with nonsmokers; plasma transport rates for apo AI, however, were similar among groups. These data suggest that the reduced HDL concentrations observed in smokers are the result of increased HDL catabolism rather than reduced HDL synthesis. Although speculative, it is possible that the cross-linking of HDL apolipoproteins we observed in our in vitro model may occur in vivo and may contribute to the reduced HDL concentrations observed in smokers.

The mechanisms for the adverse effects of gas-phase cigarette smoke on LCAT and HDL are likely to be mediated by radicals and/or reactive aldehydes (eg, acetaldehyde, acrolein, formaldehyde)12-15 generated during a cigarette's combustion. Free radical–mediated lipid peroxidation may result in the modification of lipids as well as proteins. Lipid modification results as a consequence of lipid peroxidation being a chain reaction. Protein modification, conversely, results as a consequence of lipid hydroperoxides34 or lipid hydroperoxide breakdown products35 (eg, aldehydes) forming adducts with proteins. Frei and coworkers16 used human plasma and the gas phase of cigarette smoke as a model system to examine the formation of lipid hydroperoxides in relation to the consumption of endogenous antioxidants. These investigators demonstrated that endogenous antioxidants were consumed progressively in the sequence ascorbic acid > protein thiol = albumin-bound bilirubin > uric acid > α-tocopherol. Once ascorbate is consumed, hydroperoxides (cholesteryl ester > phospholipid > triacylglycerol) begin to accumulate; this accumulation occurs despite the remaining relatively high concentrations of protein thiols, bilirubin, urate, and α-tocopherol. If, as suggested by Frei et al,16 hydroperoxide concentrations increase only after all ascorbate is consumed, it appears unlikely that smoke-induced inhibition of LCAT activity proceeds via a hydroperoxide mechanism. This conclusion is supported by our observation that the largest smoke-induced decreases in LCAT activity occur when the lowest concentrations of hydroperoxides and their breakdown products would be expected (ie, immediately after vitamin C stores are exhausted; see text and Figs 1 and 2). In addition, we have observed (unpublished observation, M.R.M. and T.M.F.) that supplementation of plasma with physiological concentrations of ascorbate (85 μmol/L) does not prevent gas-phase cigarette smoke–induced LCAT inhibition.

Direct modification of LCAT and HDL apolipoproteins in our incubations can occur with aldehydes and reactive oxygen species present in cigarette smoke. Although several amino acid residues (eg, cysteine, lysine, methionine, and tyrosine) are susceptible to modification, cysteine appears to be among the most readily modified. Interestingly, both LCAT and HDL apo AII possess cysteine groups. It has been demonstrated that the gas phase of cigarette smoke causes a reduction in plasma protein thiol groups before the complete loss of ascorbate and before detectable lipid peroxidative damage can be observed.10 Since protein thiol groups appear to be modified before detectable concentrations of lipid hydroperoxides accumulate, it is possible that inhibition of LCAT activity and protein modification of HDL proceed by mechanisms dependent on direct protein modification. The fact that we observe substantial protection of LCAT activity by addition of GSH supports this premise.

In summary, our data show that reactive components in the gas phase of cigarette smoke rapidly inhibit LCAT activity and modify HDL apolipoproteins. These smoke-induced modifications have the potential to impair the normal vascular trafficking of cholesterol and cholesteryl ester, resulting in interruption of the reverse cholesterol transport pathway. Such modification of normal HDL metabolism may be a contributing factor to increased coronary heart disease risk in smokers.

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References

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