Copper and gas-phase cigarette smoke inhibit plasma lecithin:cholesterol acyltransferase activity by different mechanisms

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Abstract Cigarette smokers have reduced levels of plasma high density lipoprotein (HDL) compared to nonsmokers and are at risk of premature cardiovascular disease. Previous work from this laboratory has shown that exposure of human plasma to gas-phase cigarette smoke (CS) inhibited the activity of lecithin:cholesterol acyltransferase (LCAT), the enzyme that catalyzes the formation of cholesteryl ester in HDL and thereby promotes HDL maturation. As CS contains free radicals that could potentially oxidize plasma lipoproteins, we examined the involvement of lipid peroxidation in LCAT inhibition. Results obtained with CS were compared with those obtained by initiating lipid peroxidation with copper ions. Exposure of dialyzed human plasma to an equivalent of one-eighth of a cigarette at 15-min intervals resulted in a progressive loss of LCAT activity (50 and 90% reductions by 1 and 6 h, respectively). A similar pattern of LCAT inhibition was produced with copper (0.5 mM) where 50 and 97% reductions were observed at 1 and 6 h, respectively. To determine whether LCAT inhibition was related to lipid peroxidation, lipoprotein fractions corresponding to VLDL-IDL, LDL, and HDL were isolated from plasma exposed to CS or copper and analyzed for changes in TBARS, the polyunsaturated fatty acid arachidonate relative to palmitate (20:4/16:0 ratio), and vitamin E concentrations. Exposure of plasma for 6 h to CS had no effect on the levels of TBARS and 20:4/16:0 ratio; however, 6 h copper treatment (0.5 mM) caused a 3.0-, 4.0-, and 1.4-fold increase in TBARS and a 17, 25, and 13% reduction in the 20:4/16:0 ratio in VLDL-IDL, LDL, and HDL fractions, respectively. In addition, a complete depletion of lipoprotein vitamin E was observed with CS, whereas copper decreased vitamin E levels by approximately 50% in each fraction. Supplementation of plasma with either vitamin C (85 μM) or butylated hydroxytoluene (BHT, 0.45 mM) was unable to protect LCAT from CS. In contrast, BHT completely protected LCAT activity from inhibition by copper. We conclude that unlike copper, CS-induced inhibition of plasma LCAT activity was unrelated to free radical-induced lipid peroxidation. The inhibition of LCAT activity by cigarette smoke may contribute to the development of atherosclerosis by impairing HDL metabolism and the reverse cholesterol transport process.—**Bielicki, J. K., M. R. McCall, J. J. M. van den Berg, F. A. Kuypers, and T. M. Forte.** Copper and gas-phase cigarette smoke inhibit plasma lecithin:cholesterol acyltransferase activity by different mechanisms. *J. Lipid Res.* 1995. **36:** 322–331.

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Elevated levels of plasma high density lipoprotein (HDL) are associated with reduced risk of atherosclerosis (1–3). The beneficial effects attributed to this lipoprotein are probably linked to the ability of HDL to remove excess cholesterol from peripheral cells (4, 5) and to facilitate transport of cholesterol to the liver: a process termed reverse cholesterol transport (6–8). A central component of the reverse cholesterol transport process is lecithin:cholesterol acyltransferase (LCAT) which catalyzes the formation of cholesteryl esters in HDL (8, 9). This esterification reaction may contribute to the efflux of cholesterol from cells by maintaining a concentration gradient for the diffusion of excess cellular cholesterol to HDL (8, 10). In the circulation, LCAT converts smaller HDL particles into larger, cholesteryl ester-rich HDL. The delivery of HDL cholesteryl esters to the liver for catabolism and subsequent excretion from the body can occur by several mechanisms including the selective uptake of cholesteryl ester independent of HDL apolipoproteins, uptake of entire HDL particles, and transfer of cholesteryl ester via cholesteryl ester transfer protein to VLDL/LDL which are cleared from the circulation via the apoB receptor pathway.

Cigarette smoking has been identified as an independent risk factor in the development of atherosclerosis (11–14), yet the underlying mechanism(s) responsible for this association is incompletely understood. Cigarette smoke contains a number of free radicals including nitric oxide, superoxide anion, and organic peroxyl radicals

Abbreviations: CS, gas-phase cigarette smoke; LCAT, lecithin:cholesterol acyltransferase; HDL, high density lipoprotein; BHT, butylated hydroxytoluene; EDTA, ethylenediamine tetraacetic acid; TBARS, thiobarbituric acid reactive substances; 20:4, arachidonic acid; 16:0, palmitic acid.

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(15), all of which have been implicated in disease pathogenesis (16–18). Yokode et al. (18) combined a cigarette smoke extract with isolated LDL and observed fragmentation of apolipoprotein B, increased electrophoretic mobility of LDL, and increased recognition of this modified lipoprotein by the scavenger receptor on macrophages. These modifications were attenuated when superoxide dismutase was included in the mixture, suggesting that superoxide anion was partially responsible for the changes in LDL. Frei et al. (19) exposed human plasma to cigarette smoke and observed lipid hydroperoxide production once endogenous vitamin C had been consumed. In addition, LDL isolated from the plasma of smokers has reduced levels of vitamin E (20) compared to the LDL of nonsmokers, suggesting that LDL of smokers may be more susceptible to oxidation. Cigarette smoke also contains a number of reactive aldehydes such as acetaldehyde, acrolein, and formaldehyde (15) which could potentially modify lipoprotein structure and electrical charge. Therefore, it is possible that constituents of cigarette smoke may contribute to the development of atherosclerosis by either facilitating lipid peroxidation or through modification of plasma lipoproteins by either free radicals or aldehydes.

Epidemiologic studies indicate that cigarette smokers have reduced concentrations of plasma HDL (12–14) and LCAT (21, 22) compared to nonsmoking individuals. Recent studies from our laboratory (23) have shown that exposure of human plasma to CS resulted in crosslinking of HDL apolipoproteins and inhibition of LCAT activity. Both of these perturbations could reduce the level of circulating HDL. Zawadzki, Milne, and Marcel (24) exposed dialyzed human plasma to copper ions and noted a shift in HDL particle size distribution and dissociation of cholesteryl ester transfer protein (CETP) from a specific subpopulation of HDL; LCAT activity, however, was not evaluated. In the present study, we sought to gain insights into the underlying mechanism whereby CS inhibited LCAT activity and whether this inhibition was linked to the peroxidation of plasma lipoproteins. To address these issues, we tested the ability of free radical scavengers, vitamin C and butylated hydroxytoluene (BHT), to protect LCAT from CS. We also monitored the generation of TBARS, changes in lipoprotein polyunsaturated fatty acid content, and vitamin E levels to assess whether any of these parameters were altered by CS in a manner consistent with the involvement of lipid peroxidation in LCAT inhibition. Cigarette smoke modification of LCAT activity was compared with copper modification as the latter is known to initiate lipid peroxidation (24, 25). The results indicated that CS inhibited LCAT by a mechanism that was unrelated to lipid peroxidation, and suggested that components of CS other than free radicals were responsible for LCAT inhibition.

MATERIALS AND METHODS

Materials

The following antioxidants were obtained from Sigma Chemical Company (St. Louis, MO): L-ascorbic acid (vitamin C, free-acid), *d,l* α-tocopherol (vitamin E), butylated hydroxytoluene (BHT), and ethylenediamine tetraacetic acid (EDTA). Lipids used as internal standards, α-tocopherol acetate and heptadecanoic acid (17:0), were also obtained from this same vendor, as were other reagents: thiobarbituric acid (TBA), 1,1,3,3-tetramethoxypropane, unesterified cholesterol, and cholesteryl oleate. Gentamicin sulfate was purchased from Gibco Laboratories (Grand Island, NY). Phosphatidylcholine (egg yolk) was from Avanti Polar Lipids Inc. (Alabaster, AL), and [4-^{14}C]cholesterol (57.1 mCi/mmol) was obtained from New England Nuclear (Claremont, CA). Hexane was HPLC-grade and was from Baxter Healthcare Corp. (McGraw Park, IL). All other organic solvents were from J. T. Baker Inc. (Phillipsburg, NJ) and of reagent grade quality. Polysilicic acid impregnated fiberglass thin-layer chromatography (TLC) sheets were from Gelman Sciences Inc. (Ann Arbor, MI).

Human plasma

Blood for each experiment was collected in EDTA from fasting, healthy, nonsmoking donors. Plasma (60–80 ml) was pooled after removal of blood cells and handled for experiments in the following manner. A sample (2.5 ml) was initially removed and served as a 4°C control; butylated hydroxytoluene (BHT, 20 μM final concentration) and gentamicin sulfate (50 μg/ml final concentration) were added to this control sample as an antioxidant and antibiotic, respectively. Initial values for TBARS, 20:4/16:0 ratios, and vitamin E concentrations were obtained from lipoproteins isolated from this nondialyzed control and were set to 100% in figures. Plasma lipid concentrations for the 16 pools used in this study were 208 ± 26, 49 ± 11, and 145 ± 41 mg/dl for total cholesterol, HDL-cholesterol, and triglycerides, respectively. Plasma was dialyzed against a 50-fold excess of phosphate-buffered saline (PBS, pH 7.4) at 4°C twice daily for 3 days to remove EDTA. Dialyzed plasma was used in these experiments because comparisons were made between CS and copper and because, in some studies, a defined quantity of vitamin C was reintroduced into plasma as an antioxidant. It should be noted that dialyzed and nondialyzed plasma behaved similarly with regard to the pattern of LCAT inhibition found upon exposure to CS as our earlier studies showing rapid inhibition of LCAT by CS were carried out with nondialyzed plasma (23). Immediately after dialysis, an aliquot (2.5 ml) of plasma was taken that served as a dialyzed control; to this material EDTA (2.72 mM final concentration), BHT, and gen-

tamicin were added. As dialysis consistently produced a 5–10% decrease in plasma LCAT activity, the rate of cholesterol esterification in dialyzed plasma was used as our t = 0 control and was set to 100% in figures.

Exposure of dialyzed human plasma to either gas-phase CS or copper

The procedure used to expose dialyzed human plasma to CS was similar to that described by Frei et al. (19). The system consisted of a 250 ml Erlenmeyer side-arm flask to which 12–15 ml of plasma was added. Either air (control) or an equivalent of one-eighth of a cigarette (University of Kentucky 2R1 research cigarettes) was introduced into the flask every 15 min for 6 h. Cambridge filters were used to filter particulates (particles $> 0.01\ \mu m$) from CS entering the flask. Incubations were performed at 37°C, and at given time-points, samples of plasma were removed and chilled to 4°C. EDTA and BHT were added to prevent oxidation during sample analysis.

In parallel incubations, lipid peroxidation was initiated by adding copper to plasma (0.5 mM, final concentration). At given time-points, an aliquot of plasma from either control incubations or copper-treated plasma was removed; BHT and EDTA were added to prevent further oxidation.

LCAT activity measurements

LCAT activity was measured by the exogenous substrate method described by Chen and Albers (26). A 7.5-μl aliquot of dialyzed control plasma or plasma exposed to either air, CS, or copper was added to a reaction mixture of 0.25 ml containing the following constituents: 20 mM Tris-HCl (pH 8.0), 0.15 M NaCl, 0.27 mM EDTA, 0.5% human serum albumin, 5 mM β-mercaptoethanol, and proteoliposomes composed of [^{14}C]cholesterol, human apoA-I, egg yolk phosphatidycholine, and unlabeled cholesterol. Esterification of [^{14}C]cholesterol was assessed after 30 min of incubation at 37°C. By using this proteoliposome assay, we were able to monitor decreases in plasma LCAT activity resulting from modifications of the enzyme rather than changes in the endogenous substrate (HDL).

Lipoprotein isolation

Lipoproteins within density intervals of <1.019, 1.019–1.063, and 1.063–1.21 g/ml were obtained from plasma by sequential preparative ultracentrifugation and corresponded to VLDL-IDL, LDL, and HDL, respectively (27). Density was adjusted with solid NaBr and all centrifugation steps were performed at 4°C. Lipoprotein fractions were dialyzed into saline–EDTA; gentamicin was added to dialyzed samples.

Electrophoresis and chemical analyses

Protein was measured by the method of Markwell et al. (28), and bovine serum albumin was used as the standard. Changes in apolipoproteins within each lipoprotein fraction as induced by CS or copper were evaluated by 4–20% non-reducing SDS-PAGE (29) using Schleicher and Schuell precast minigels. Plasma was analyzed for changes in lipoprotein electrical charge using Paragon agarose gels (Beckman). Thiobarbituric acid reactive substances (TBARS) were measured by the procedure described by Kosugi et al. (30). The ratio of arachidonic acid to palmitic acid (20:4/16:0) was quantitated by gas–liquid chromatography as described by Muskiet et al. (31) using heptadecanoic acid (17:0) as an internal standard. Vitamin E levels were determined by the method of van den Berg et al. (32), and α-tocopherol acetate was used as an internal standard.

RESULTS

Inhibition of plasma LCAT activity by either CS or copper

Figure 1A reveals that in dialyzed human plasma, LCAT activity was very sensitive to CS. A 20% decrease in activity was observed after 15 min, and by 1 h of exposure to CS, approximately 50% of the LCAT activity was lost; further exposures resulted in up to a 90% loss of activity compared to controls by 6 h. To determine the concentration of copper required to inhibit LCAT activity, plasma was exposed to 0.2, 0.4, and 0.5 mM copper for up to 6 h as seen in Fig. 1B. Like CS, 0.5 mM copper treatment resulted in a rapid inhibition of LCAT activity where there was a 23% decrease in 15 min and almost complete inhibition at 6 h; hence, this concentration was used in all subsequent studies.

Changes in apolipoproteins after exposure of dialyzed plasma to CS or copper

Exposure of plasma to CS resulted in crosslinking of HDL apolipoproteins as seen in **Fig. 2,** lane 10. The formation of higher molecular weight forms of apolipoproteins A-I and A-II were verified by Western blot analysis (data not shown), and these changes were similar to those previously described by McCall et al. (23). Plasma exposed to CS for 6 h showed a 30% and 12% increase in agarose-gel mobility of LDL and HDL, respectively (data not shown). Treatment of plasma with 0.5 mM copper for 6 h, on the other hand, showed either very little or no crosslinking of HDL apolipoproteins (Fig. 2, lane 9) and no increases in lipoprotein electrophoretic mobility in agarose (data not shown). Neither fragmentation nor intermolecular crosslinking of apoB was observed after CS or copper exposures suggesting that HDL apolipoproteins were uniquely sensitive to constituents of CS.

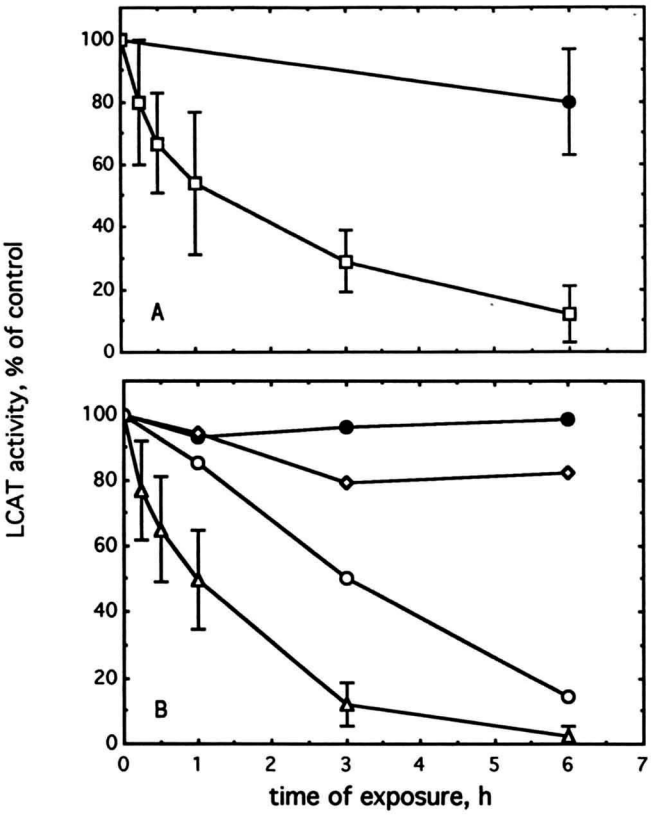

Fig. 1. Inhibition of plasma LCAT by gas-phase cigarette smoke (CS) and copper ions. Dialyzed human plasma (15 ml) was exposed to either an equivalent of one-eighth of a cigarette every 15 min for 6 h (A) or 0.5 mM copper (B). At the time-points indicated, aliquots of plasma (0.5 ml) were removed and EDTA and BHT were added as described in Methods. LCAT activity was measured by an exogenous substrate method (23) using proteoliposomes containing [^{14}C]cholesterol. The results were expressed as a percentage of control LCAT activity in dialyzed plasma (mean rate of cholesterol esterification equaled 8.1 ± 1.3%/0.5 h). Closed circles (●) denote controls; squares (□) plasma exposure to CS, and diamonds (◇), open circles (○), and triangles (△) plasma exposed to 0.2, 0.4, and 0.5 mM copper ions, respectively. Three separate experiments using different batches of pooled plasma were performed comparing air, smoke, and 0.5 mM copper (means ± SD are shown). The data for 0, 0.2, and 0.4 mM copper are from a single experiment, and measurements of LCAT activity were in duplicate.

Lipoprotein TBARS and 20:4/16:0 fatty acid ratios after exposure of dialyzed plasma to CS or copper

To determine whether lipid peroxidation paralleled LCAT inhibition, lipoproteins were isolated from dialyzed human plasma that had been exposed to either air, CS, or copper, and lipid peroxidation was assessed by TBARS formation and by changes in fatty acid composition. The loss of polyunsaturated fatty acid was assessed by determining the ratio of the major polyunsaturated fatty acid, arachidonate (20:4), to the major saturated fatty acid, palmitate (16:0). As shown in **Table 1** compared to air controls, CS failed to produce changes in lipoprotein TBARS suggesting that alterations in HDL apolipoproteins were not the result of free radical-induced lipid peroxidation. Exposure to 0.5 mM copper produced a substantial time-dependent rise in TBARS within the lipid-rich lipoproteins including VLDL-IDL and LDL which reached 3- to 4-times control levels by 6 h. Relatively less TBARS were recovered in the protein-rich HDL fraction. TBARS may have been underestimated as a loss of water-soluble aldehydes (malondialdehyde) may have occurred during isolation of lipoproteins. The increase in TBARS obtained with copper, however, suggested that, unlike CS, copper stimulated the oxidation of lipoprotein polyunsaturated fatty acids. To examine this possibility, the ratio of 20:4 to 16:0 within lipoprotein fractions was assessed. Arachidonate concentrations were determined because this major fatty acid is highly susceptible to oxidation relative to the saturated fatty acid, palmitate, which is not. Decreases in the 20:4/16:0 ratio, therefore, reflect oxidation of lipoprotein polyunsaturated fatty acids. As seen in **Fig. 3,** copper treatment caused 17, 25, and 13% reductions in this ratio by 6 h in VLDL-IDL, LDL, and HDL fractions, respectively. Thus, the addition of 0.5 mM copper to plasma produced low levels of lipid peroxidation. In contrast, the lipoprotein 20:4/16:0 ratio was unaltered by CS exposures. These data suggested that CS-induced inhibition of plasma LCAT activity was unrelated to lipid peroxidation and the breakdown of lipoprotein polyunsaturated fatty acids.

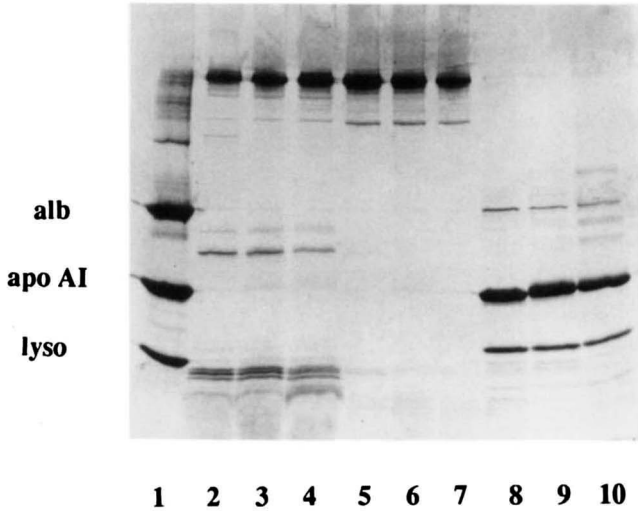

Fig. 2. SDS-PAGE analysis of lipoproteins isolated from dialyzed plasma exposed to CS or copper. Dialyzed human plasma was exposed to either one-eighth of a cigarette every 15 min for 6 h or to 6 h copper (0.5 mM). Electrophoretic patterns are as follows: lane 1 molecular weight standards; lanes 2, 3, and 4, VLDL-IDL from air, copper, or CS exposures, respectively; lanes 5, 6, and 7, LDL from air, copper, or CS exposures, respectively; lanes 8, 9, and 10, HDL from air, copper, or CS exposures, respectively. The additional high molecular weight bands in lane 10 are the result of crosslinking of apolipoproteins on HDL.

TABLE 1. TBARS from lipoprotein fractions isolated from dialyzed human plasma that had been exposed to either air, gas-phase cigarette smoke, or copper

Lipoprotein Fraction	Exp. #	Treatment	Time 1 h	Time 3 h	Time 6 h
			nmol MDA equivalents/mg protein		
VLDL-IDL[a]	1	air[b]	3.0	2.1	2.9
		smoke[b]	5.4	2.0	2.3
		copper[c]	5.3	7.7	11.4
VLDL-IDL	2	air	8.4	7.2	7.3
		smoke	8.9	7.8	7.5
		copper	12.0	16.3	24.4
LDL	1	air	1.0	0.8	0.8
		smoke	0.7	0.9	0.9
		copper	1.5	3.3	5.9
LDL	2	air	2.1	2.1	2.4
		smoke	2.5	2.2	2.6
		copper	2.8	6.3	8.9
HDL	1	air	0.5	0.4	0.3
		smoke	0.4	0.4	0.7
		copper	0.5	0.5	0.9
HDL	2	air	1.2	1.1	1.2
		smoke	1.2	1.5	1.8
		copper	1.2	1.3	1.7

[a]Values for lipoproteins isolated from 4°C plasma were as follows: VLDL-IDL, 3.0 and 11.5; LDL, 1.2 and 2.6; HDL, 0.6 and 1.3 nmol MDA equivalents/mg protein for experiments 1 and 2, respectively.
[b]Dialyzed human plasma was exposed to either air or gas-phase CS every 15 min over the time period indicated.
[c]Final concentration was 0.5 mM.

Lipoprotein vitamin E levels after exposure of dialyzed plasma to CS or copper

Vitamin E is a lipid-soluble antioxidant that is known to protect lipoproteins from peroxidation. As the consumption of this antioxidant has been shown to precede fatty acid breakdown (33), we measured the level of lipoprotein vitamin E after exposure of dialyzed plasma to either CS or copper to assess oxidation. Copper treatment caused a 40–60% reduction in the concentrations of vitamin E by 6 h (**Fig. 4**). This decrease paralleled the changes in TBARS and lipoprotein 20:4/16:0 ratio (Table 1 and Fig. 3). Surprisingly, the time-course of vitamin E depletion with CS exposure was considerably more rapid than that observed with copper. Exposure of dialyzed human plasma to CS resulted in greater than 60% depletion of lipoprotein vitamin E by 3 h and complete depletion by 6 h. In addition, the pattern for vitamin E depletion by CS was very similar to that observed for LCAT inhibition suggesting a possible link between these two parameters.

Effect of antioxidants on CS-induced changes in LCAT activity and HDL vitamin E

To determine whether both LCAT inhibition and depletion of lipoprotein vitamin E by CS occurred by similar mechanisms, we supplemented dialyzed human plasma with 85 μM vitamin C prior to each exposure of CS. Vitamin C was used because Kagan et al. (34) have shown that vitamin C can recycle tocopherol radicals to regenerate the reduced form of vitamin E. As shown in **Fig. 5A,** repeated additions of vitamin C spared HDL vitamin E from oxidation by CS. In contrast, vitamin C was ineffective at protecting LCAT (Fig. 5B). These data suggested that CS-induced changes in plasma vitamin E levels may be mediated by free radicals, whereas LCAT inhibition probably occurred via a mechanism that was unrelated to free radicals and lipid peroxidation. This conclusion was supported by data obtained in other experiments in which plasma was supplemented with 1 mM vitamin C prior to each CS exposure and similar results were obtained. In other studies (data not shown), supplementation of plasma with 0.25 mM vitamin E also did not protect LCAT from CS.

Butylated hydroxytoluene (BHT) is an effective free radical quenching agent often used as a preservative to protect lipids from oxidation. Therefore, we supplemented plasma with this antioxidant to assess whether LCAT activity was protected from either CS or copper. **Fig. 6A** shows that the presence of BHT (0.45 mM) could not protect LCAT from the deleterious effects of CS. With copper, however, BHT completely preserved plasma LCAT activity (Fig. 6B). The latter observation is consistent with the premise that copper, unlike CS, inhibited LCAT by a mechanism that involved free radicals and free radical-induced lipid peroxidation.

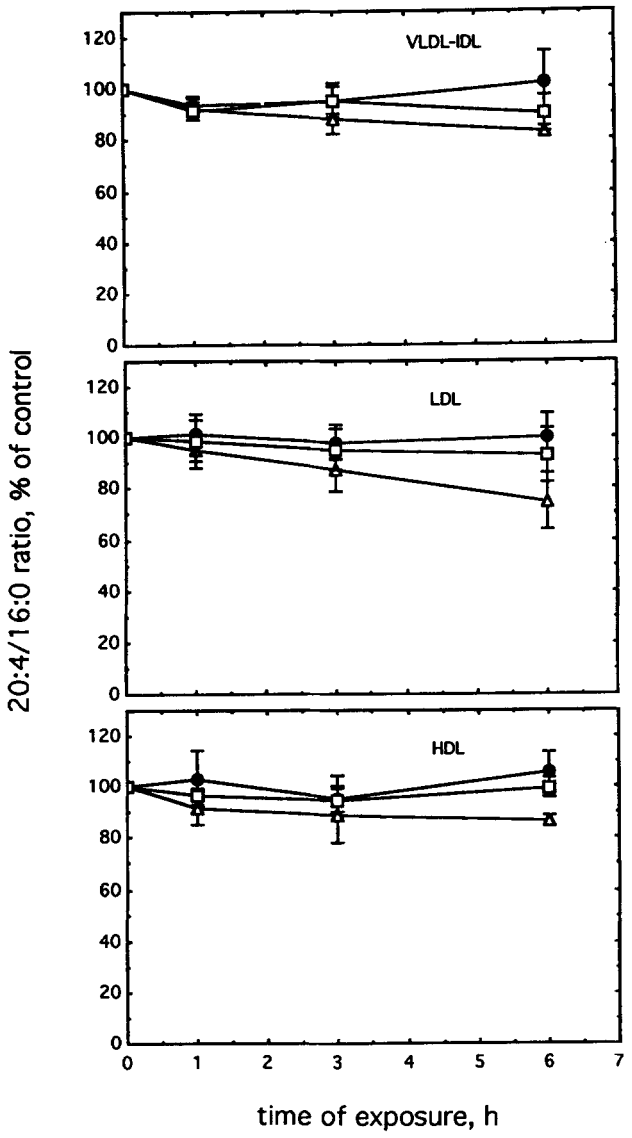

Fig. 3. Effect of either gas-phase CS or copper ions on the polyunsaturated fatty acid content of plasma lipoproteins. Dialyzed human plasma (15 ml) was exposed to either an equivalent of one-eighth of a cigarette every 15 min for 6 h or copper (0.5 mM). At the time-points indicated, aliquots of plasma (2.5 ml) were removed and EDTA and BHT were added as described in Methods. The concentration of arachidonic acid was used as a measure of lipid peroxidation, and these values were normalized to the amount of palmitate recovered in each sample. Values shown are percentages of control ratios which were 0.164 ± 0.008, 0.365 ± 0.035, and 0.446 ± 0.048 for VLDL-IDL, LDL, and HDL, respectively. Circles (●) denote air controls; squares (□) plasma exposed to CS, and triangles (△) plasma exposed to copper ions. Values are from three separate experiments each performed with different batches of pooled plasma. Means ± SD are shown (decreases with copper at 6 h were statistically different from controls, $P < 0.05$, two group unpaired *t*-test).

DISCUSSION

We found that in dialyzed human plasma, CS rapidly inhibits LCAT activity. The present results corroborate and expand our previous report showing a rapid decrease in LCAT activity when non-dialyzed plasma was exposed to CS (23). We also found that addition of copper ions to plasma inhibited LCAT activity. With 0.5 mM copper, the kinetics for LCAT inhibition were similar to the time-course of inhibition observed with CS. Yet, the addition of EDTA to plasma was unable to protect LCAT from CS, indicating that copper, if present in trace quantities, was not responsible for LCAT inhibition by CS.

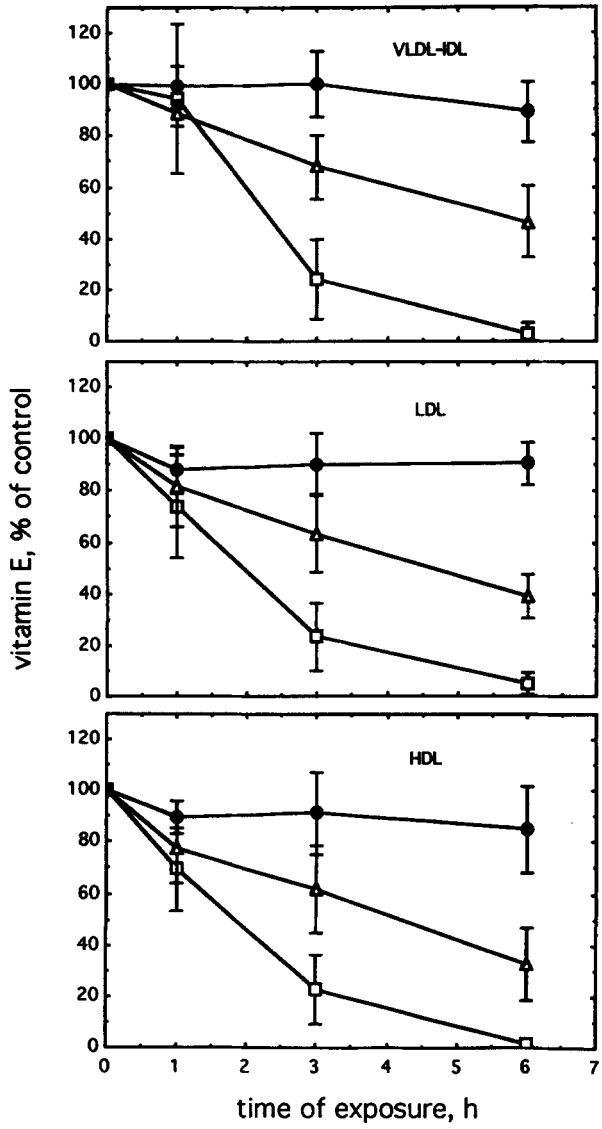

Fig. 4. Effect of either gas-phase CS or copper ions on lipoprotein vitamin E. Dialyzed human plasma (15 ml) was exposed to either an equivalent of one-eighth of a cigarette every 15 min for 6 h or copper (0.5 mM). At the time-points indicated, aliquots of plasma (2.5 ml) were removed and EDTA and BHT were added as described in Methods. Vitamin E mass was normalized to the amount of protein recovered in each sample. Values shown are percentages of control vitamin E levels which were 60.3 ± 22.6, 15.8 ± 2.7, and 5.3 ± 1.2 nmol vitamin E/mg protein for VLDL-IDL, LDL, and HDL, respectively. Circles (●) denote air controls; squares (□) plasma exposed to CS, and triangles (△) plasma exposed to copper ions. Values are from three separate experiments each performed with different batches of pooled plasma. Means ± SD are shown.

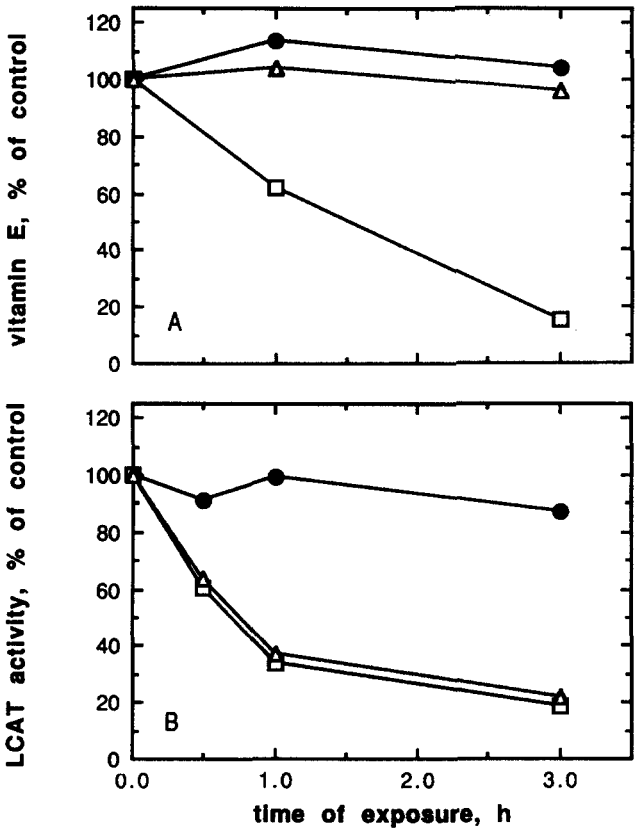

Fig. 5. Maintenance of HDL vitamin E levels by vitamin C and the failure of this supplementation to protect LCAT activity from CS. Vitamin C (85 μM, final concentration) was added to dialyzed human plasma (15 ml) prior to each exposure of CS (i.e., every 15 min). At the time-points indicated, aliquots of plasma (2.5 ml) were removed for isolation of HDL by ultracentrifugation and vitamin E quantification (panel A). Vitamin E levels were normalized to the amount of protein recovered in each sample. Values shown are percentages of a control that was 4.43 nmol vitamin E/mg protein. Panel B shows the effect of vitamin C additions on LCAT activity. Values shown are percentages of control LCAT activity (10.4% esterification of [^{14}C]cholesterol in 0.5 h). Circles (●) denote plasma exposed to air; squares (□) plasma exposed to CS, and triangles (△) plasma supplemented with vitamin C and exposed to CS. Values shown are from one of two experiments in which similar results were obtained; LCAT activity was measured in duplicate.

Although CS and copper both inhibited LCAT activity, HDL isolated from plasma incubations showed different properties on SDS and agarose gels depending on the oxidant. Similar to the previous report of McCall et al. (23), CS exposures produced crosslinking of HDL apolipoproteins and increased the negative charge of plasma lipoproteins. Crosslinking of HDL apolipoproteins may produce conformational changes that alter the net negative charge of HDL. With copper treatment, however, apolipoprotein crosslinking was not apparent. These results are consistent with the inability of copper to alter the electrophoretic mobility of plasma lipoproteins, and suggested that the peroxidation observed with copper was insufficient to alter the electrical charge of plasma lipoproteins. The presence of copper-binding proteins in plasma may have reduced the effective concentration of free copper ions and thereby limited the extent of lipid peroxidation in our system.

Cigarette smoke has been shown to initiate lipid peroxidation in plasma (19). However, we found that exposure of dialyzed plasma to CS, unlike copper, was not associated with an increase in lipoprotein TBARS and decrease in 20:4/16:0 ratio. In addition, vitamin C and BHT were unable to protect LCAT activity and HDL apolipoproteins (data not shown) from gas-phase CS. As these antioxidants are capable of quenching most free radicals, it is unlikely that free radicals in CS were responsible for LCAT inhibition. The inability of vitamin C and BHT to protect LCAT does not completely exclude free radical involvement in CS-induced LCAT inhibition. It is still possible that a long-lived, not very reactive, free radical spe-

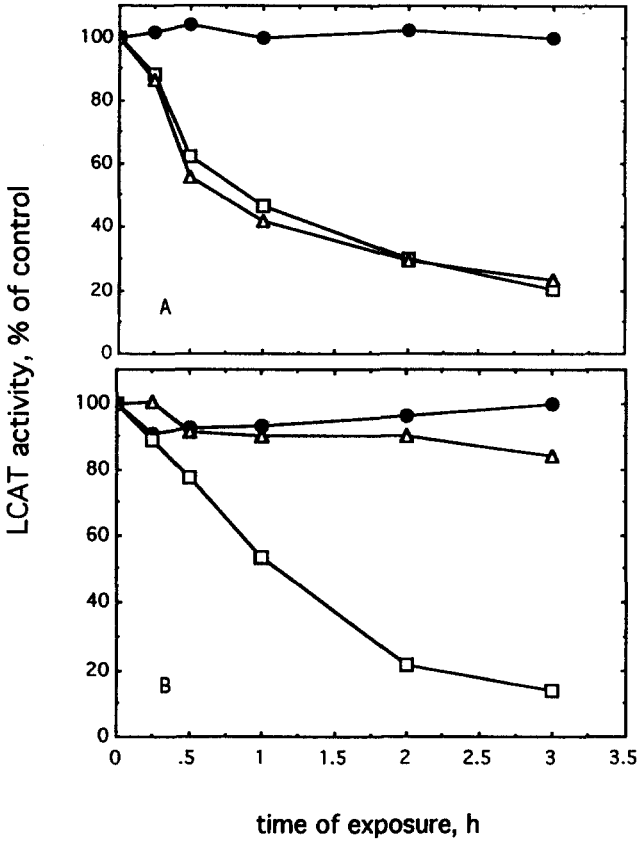

Fig. 6. Effects of BHT on CS- and copper-mediated inhibition of LCAT. Dialyzed human plasma was exposed to either an equivalent of one-eighth of a cigarette every 15 min for 3 h (A) or 0.5 mM copper (B). At the time-points indicated, aliquots of plasma (0.1 ml) were removed and LCAT activity was measured. Values shown are percentages of control LCAT activity (8.9% esterification of [^{14}C]cholesterol in 0.5 h). Circles (●) denote controls; squares (□) plasma exposed to CS (A) or copper ions (B), and triangles (△) plasma supplemented with BHT (0.45 mM) and then exposed to CS (A) or copper ions (B). Values are from a single experiment with pooled plasma; LCAT activity was measured in duplicate.

cies may directly modify LCAT. Our results do indicate, however, that free radical-induced lipid peroxidation and the subsequent formation of lipid breakdown products were not responsible for LCAT inhibition. This latter conclusion is substantiated by the inability of CS to facilitate the breakdown of lipoprotein polyunsaturated fatty acids. Thus, during the time-course of these experiments, LCAT activity was rapidly inhibited before the onset of lipid decomposition. These findings indicate that changes in agarose gel mobility of plasma lipoproteins, HDL apolipoprotein structure, and LCAT activity probably occurred by a mechanism that was not related to lipid peroxidation, but rather may have occurred via direct chemical modification of proteins by constituents of CS.

McCall et al. (23) have found that reduced glutathione partially protected LCAT activity from CS, and we have since observed that glutathione can also protect HDL from crosslinking (data not shown). Cigarette smoke contains high concentrations of aldehydes such as acetaldehyde, acrolein, and formaldehyde (15), and it has been reported that acrolein can react with thiols (35). Reznick et al. (36) found that incubation of plasma with gas-phase CS inhibited creatine kinase activity, but not lactate dehydrogenase, aspartate aminotransferase, and glutamyl transferase indicating that exposure of plasma to CS does not produce a general, nonspecific inhibition of enzymes. In other experiments, Reznick et al. (36) found that a mixture of aldehydes found in cigarette smoke also inhibited creatine kinase. Furthermore, these investigators provided evidence that CS-induced inhibition of creatine kinase was related to modifications of its protein sulfhydryl groups. LCAT contains two reduced cysteine residues at positions 31 and 184 of the primary amino acid sequence (9, 37). These free sulfhydryl groups are thought to be in close proximity to the active site of the enzyme. Site-directed mutagenesis studies have revealed that inhibitors of LCAT modify these residues and sterically block the active site of LCAT (37). Therefore, it is possible that components of CS, perhaps aldehydes, covalently modify cysteine residues to produce LCAT inhibition.

The inhibition of LCAT activity by copper may work by a different mechanism than CS. As pointed out earlier, incubation of plasma with copper ions resulted in an increase in TBARS and a reduction in the ratio of 20:4/16:0 within plasma lipoproteins. Copper can facilitate the formation of lipid radicals, and these free radicals may directly inhibit LCAT. Furthermore, free radicals facilitate lipid peroxidation which leads to the breakdown of polyunsaturated fatty acids and the formation of reactive aldehydes such as malondialdehyde and 4-hydroxynonenal (33). Such aldehydes can potentially modify LCAT as it has been shown that they alter lipoprotein structure and metabolism (38–40). The hypothesis that a lipid peroxidation product can inactivate LCAT is supported by our experimental finding that the lipophilic antioxidant BHT completely protected against copper-induced inhibition of LCAT activity. In addition, LDL isolated from plasma exposed to copper was found to inhibit LCAT suggesting that the LCAT inhibitory factor resided with the lipoprotein fraction (unpublished observation). Alternatively, copper may directly interact with LCAT to produce an inhibition in enzymatic activity. Parthasarathy (25) and others (41, 42) have shown that copper can radicalize sulfhydryl compounds to produce thiyl radicals that promote the oxidation of LDL. As LCAT contains two free thiols (amino acids 31 and 184) near its active site, the oxidation of these residues may interfere with the enzymes catalytic activity.

Unexpectedly, we found that CS completely depleted lipoprotein vitamin E concentrations by 6 h, yet this depletion was unrelated to changes in polyunsaturated fatty acids. In contrast, copper reduced vitamin E and polyunsaturated fatty acid concentrations. The reason for this difference between CS and copper is unknown but may reflect differences in the site of action between CS and copper. CS contains a wide variety of reactive compounds which, in our system, can partition into the aqueous phase of plasma. These radicals, including nitric oxide and organic peroxyl radicals, may preferentially oxidize vitamin E at the surface of lipoprotein particles but during the same time course may be insufficient to stimulate the formation of lipid hydroperoxides. Copper, on the other hand, is thought to initiate the breakdown of polyunsaturated fatty acids by facilitating the production of lipid radicals from preformed lipid peroxides associated with fatty acyl chains within the lipoprotein surface and core. Thus, the differences between CS and copper with regard to the reductions in vitamin E and 20:4/16:0 ratios may reflect differences in their initial sites of action.

As stated above, the depletion in lipoprotein vitamin E that we observed with CS was prevented when dialyzed plasma was supplemented with vitamin C, suggesting that plasma vitamin C concentrations may be an important determinant of lipoprotein vitamin E status as proposed by Kagan et al. (34). This being the case, the observations that smokers have reduced levels of vitamin C (43–45), compared to nonsmoking controls, would lend support to the hypothesis that CS may adversely effect lipoprotein vitamin E concentrations. The interaction between these and other antioxidants may partially explain why conflicting reports (20, 43) have appeared in the literature on the effects of CS on plasma vitamin E levels in vivo.

Although it is well documented that CS is associated with reduced HDL-cholesterol concentrations and an increased risk of coronary heart disease, the in vivo mechanism accounting for these changes remains controversial. Moriguchi et al. (46), for example, have demonstrated a decrease in the level of plasma HDL_2 in smokers while Haffner et al. (22) reported that only the HDL_3 fraction was affected. These differences may reflect a dichotomy in

the populations examined and/or the relatively small number of subjects participating in these studies. Albers et al. (21) and Haffner et al. (22) observed a decrease in LCAT mass in cigarette users compared to nonusers. In contrast, Moriguchi et al. (46) suggested that plasma LCAT activity tended to be lower in smokers (as stated by the authors), but the sample size was too small to show statistical significance. Parscau and Fielding (47) found no difference in plasma LCAT activity between smokers and nonsmokers, but only 10 healthy subjects were examined in each group. Larger, population-based studies are required to determine whether acute exposures to cigarette smoke decreases plasma LCAT activity in vivo.

Our study confirms the previous observation that plasma LCAT activity and HDL-apolipoproteins are highly susceptible to modification by CS (23), and, in addition, provides evidence that components of CS other than free radicals are responsible for these modifications. The alterations in LCAT and HDL that we observed were probably linked to protein modifications rather than to the formation of lipid hydroperoxide breakdown products. Crosslinking of HDL apolipoproteins and inhibition of LCAT activity, if occurring in vivo, could contribute to the low levels of HDL in smokers and may provide a mechanistic link between premature cardiovascular disease and cigarette use.

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