Gas phase oxidants of cigarette smoke induce lipid peroxidation and changes in lipoprotein properties in human blood plasma

Protective effects of ascorbic acid

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Cigarette smoke (CS) is known to contain a large number of oxidants. In order to assess the oxidative effects of CS on biological fluids, we exposed human blood plasma to filtered (gas phase) and unfiltered (whole) CS, and determined the rate of utilization of endogenous antioxidants in relation to the appearance of lipid hydroperoxides. Lipid peroxidation was measured with a specific and sensitive assay that can detect lipid hydroperoxides at plasma levels as low as 10 nM. We found that exposure of plasma to the gas phase of CS, but not to whole CS, induces lipid peroxidation once endogenous ascorbic acid has been oxidized completely. In addition, CS exposure caused oxidation of plasma protein thiols and albumin-bound bilirubin, whereas uric acid and α-tocopherol were not consumed at significant rates. In plasma exposed to the gas phase of CS, low-density lipoprotein exhibited slightly increased electrophoretic mobility, but there was no apparent degradation of apolipoprotein B. Our results support the concept of an increased vitamin C utilization in smokers, and suggest that lipid peroxidation induced by oxidants present in the gas phase of CS leads to potentially atherogenic changes in lipoproteins.

INTRODUCTION

Cigarette smoke (CS) has been implicated as a major risk factor in chronic obstructive pulmonary diseases such as chronic bronchitis and emphysema, in chemical carcinogenesis and in atherosclerotic arterial disease [1–3]. The mechanisms of the adverse biological effects of CS appear to include oxidative damage to essential biological constituents, e.g. inactivation of antiproteinases both by oxidants present in the CS itself [4,5] and by oxidants generated by CS-induced activation of endogenous phagocytic cells [6–8]. It is known that CS also increases the number of phagocytes in blood [9] and lungs [5,10], and decreases plasma levels of high-density lipoprotein (HDL) [11,12]. Since lipid peroxidation in low-density lipoprotein (LDL) is thought to play a pivotal role in atherogenesis [13,14], oxidation of LDL might be an important mechanism whereby CS can accelerate atherogenesis.

It has been shown that incubation of isolated human LDL with a filtered aqueous extract of CS leads to changes in the electrophoretic mobility of LDL, extensive fragmentation of apolipoprotein B (apo B) and enhanced uptake of LDL by macrophages [15]. These potentially atherogenic effects of CS on LDL can be partially inhibited by the antioxidant enzyme superoxide dismutase. Interestingly, no increase in thiobarbituric-acid-reactive substances (TBARS) could be demonstrated in LDL after exposure to CS extract, suggesting that no lipid peroxidation had occurred. Likewise, Harats *et al.* [16] could find no differences in the levels of TBARS in freshly prepared plasma or LDL of smokers as compared with non-smokers. However, they found that LDL from smokers was more susceptible than LDL from non-smokers to peroxidative modification by cultured aortic smooth muscle cells. In contrast, using the same assay for lipid peroxidation, Lentz & DiLuzio [17] reported increases in levels of TBARS in rabbit pulmonary alveolar macrophages exposed *in vitro* to a filtered aqueous extract of CS. Thus experimental evidence for CS-induced lipid peroxidation is mixed and contradictory, probably because the TBARS test is inaccurate and non-specific [18,19].

We have recently developed a sensitive and selective h.p.l.c. assay with post-column chemiluminescence detection that allows direct measurement of lipid hydroperoxides in biological fluids at concentrations as low as 10 nM [20,21]. This assay has been successfully used for investigation of antioxidant defences and lipid peroxidation in plasma exposed to various types of oxidant stress, including aqueous peroxyl radicals and activated phagocytes [22–25].

In the present paper we have characterized the action of CS upon lipoproteins in whole plasma in relation to the ability of plasma antioxidants to prevent CS-induced oxidative damage. We show that acute exposure to the gas phase of CS induces peroxidation of plasma lipids as detected by the h.p.l.c./chemiluminescence assay, and that ascorbic acid appears to be the only endogenous antioxidant in plasma that can completely protect the lipids against detectable peroxidative damage under these conditions. We have also observed changes in the properties of the plasma lipoproteins that may render them atherogenic.

MATERIALS AND METHODS

Materials

The cigarettes used in this study were University of Kentucky (UK) 2R1 standard cigarettes containing 23 mg of tar and 2.2 mg of nicotine per cigarette (according to the Federal Tobacco Council). Filters were standard Cambridge filters rated to remove 99.9% of all particles $> 0.01\ \mu m$ in diameter [26]. Sodium heparin vacutainers (143 units for 10 ml of blood) were purchased

Abbreviations used: apo B, apolipoprotein B; CS, cigarette smoke; HDL, high-density lipoprotein; IDL, intermediate-density lipoprotein; LDL, low-density lipoprotein; TBARS, thiobarbituric-acid-reactive substances; VLDL, very-low-density lipoprotein.

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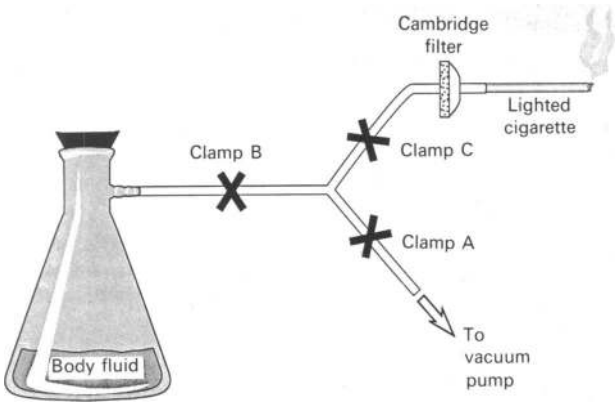

Fig. 1. Scheme of CS exposure system

Clamp C is closed and clamps B and A are opened until a partial vacuum is established in the flask. Clamp A is then closed and clamp C slowly opened to allow a puff of smoke to enter the flask. Clamp B is then closed and the flask is incubated in a metabolic shaker at 37 °C.

from Becton–Dickinson (Rutherford, NJ, U.S.A.). L-Ascorbic acid (vitamin C) was obtained from Fisher (Fair Lawn, NJ, U.S.A.), and 6-amino-2,3-dihydro-1,4-phthalazinedione (isoluminol), microperoxidase (type MP-11), 5,5′-dithiobis(2-nitrobenzoic acid) and ascorbate oxidase from Cucurbita species were from Sigma (St. Louis, MO, U.S.A.). Ion-pair cocktail Q12 (0.5 M solution of dodecyltriethylammonium phosphate) was obtained from Regis (Morton Grove, IL, U.S.A.). All other chemicals used were of the highest purity commercially available. H.p.l.c. equipment, u.v. and chemiluminescence detectors, and computer systems for data acquisition and reprocessing were the same as described previously [21]. All h.p.l.c. analytical columns used were purchased from Supelco (Bellefonte, PA, U.S.A.), and were of the dimensions 25 cm × 4.6 mm internal diam. Each analytical column was preceded by a guard column (2 cm × 4.6 mm internal diam.) containing the same material as the analytical column. The guard columns were also obtained from Supelco.

Blood collection and exposure of plasma to CS

Blood from five normolipidaemic male donors, 31–54 years of age, was drawn into sodium heparin vacutainers and centrifuged immediately at 1000 *g* and 4 °C for 10 min. Ranges of plasma lipid values were determined, and found to be as follows: triacylglycerols, 29–37 mg/dl; total cholesterol, 147–214 mg/dl; HDL-cholesterol, 44–65 mg/dl. For experiments, 20 or 40 ml of plasma was placed into a 250 or 500 ml filter flask respectively. After pre-incubation in a metabolic shaker at 37 °C for 5 min, a first set of samples was withdrawn for determination of lipid hydroperoxides and antioxidants (time 0), and the plasma was subsequently exposed to CS. The side-arm of the filter flask was connected via a plastic Y connector to a vacuum and to a UK 2R1 standard cigarette with or without a Cambridge filter system, as schematically depicted in Fig. 1. The flask was evacuated to 0.2 kPa, and then the connector to the vacuum was clamped. The cigarette was lit, and 25–50 ml of whole or gas-phase (filtered) CS was slowly introduced into the flask, burning about one-eighth of the cigarette. The side-arm to the cigarette was clamped and the flask was put into a metabolic shaker at 37 °C for 20 min. At the end of the incubation time plasma was sampled and another 'puff' of CS was introduced into the flask as described above. With whole blood instead of plasma in the flasks, 6 puffs delivered over 120 min increased the carbon monoxyhaemoglobin level of the blood to approx. 75%. Control plasma samples were incubated in similar flasks at 37 °C, but with puffs of sham room air exposure instead of CS.

Quantification of lipid hydroperoxides

The various classes of lipid hydroperoxides were determined with an ultrasensitive h.p.l.c./isoluminol chemiluminescence assay, as described previously [20,21]. This assay has a detection limit for lipid hydroperoxides of 10 nM in plasma, and measures the hydroperoxy groups themselves, rather than indirect indices of lipid peroxidation such as diene conjugation or TBARS.

Quantification of ascorbic acid and uric acid

The levels of ascorbic acid and uric acid were determined by paired-ion reversed-phase h.p.l.c. coupled with electrochemical detection, as described previously [23].

Quantification of α-tocopherol and bilirubin

The concentrations of these antioxidants in plasma were determined as described previously by h.p.l.c., with detection at 210 and 460 nm respectively [22].

Quantification of protein thiols

The concentration of the plasma proteins' thiol groups was determined in 100 μl plasma samples, using 5,5′-dithiobis(2-nitrobenzoic acid) to derivatize the thiol groups, with subsequent spectrophotometric measurement at 412 nm as described by Ellman [27].

Characterization of lipoproteins

At the termination of the experiment, plasma samples were placed on ice and the electrophoretic migration of lipoproteins was examined (within 1–2 h) by agarose gel electrophoresis using Beckman Paragon Lipid gels run according to the manufacturer's recommendation. Gels were stained with fat 7B stain. Dried gels were scanned with a RFT densitometer.

To evaluate changes in LDL particle size distribution after exposure to CS, plasma control and experimental samples were electrophoresed on precast 2–16% non-denaturing polyacrylamide gradient gels (Pharmacia, Piscataway, NJ, U.S.A.), as described by Krauss & Burke [28]. Particle sizes were determined by use of a standard curve constructed using proteins with known molecular diameters (Pharmacia, high-molecular-mass kit) and carboxylated latex beads (Dow Chemical, Indianapolis, IN, U.S.A.). Gels were stained with Oil Red O and scanned with the RFT densitometer.

SDS/PAGE was used to assess degradation of LDL apo B. LDL was first isolated at $d = 1.019$–1.063 by sequential ultracentrifugation as described by Lindgren [29]. Apo B molecular mass was determined on 4–20% polyacrylamide gels in the presence of 1.0% SDS.

RESULTS

Fresh human plasma was exposed to the gas phase of CS from a UK 2R1 standard cigarette, using a Cambridge filter system to remove virtually all particles > 0.01 μm in diameter contained in whole smoke. The consumption of selected plasma antioxidants was measured in relation to the appearance of hydroperoxides formed from endogenous lipids. Ascorbic acid was the first antioxidant to be consumed (Fig. 2), its concentration dropping from 45 μM to < 1 μM after two puffs. Protein thiols and albumin-bound bilirubin were also oxidized at significant rates from the beginning of the experiment, while oxidation of uric acid and α-tocopherol was very slow. Lipid peroxidation began after the complete consumption of ascorbic acid (Fig. 2). Three different classes of lipid hydroperoxides were formed simultaneously, i.e.

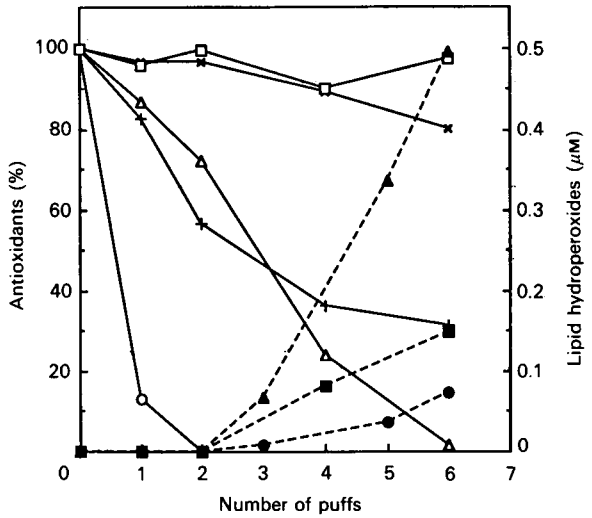

Fig. 2. Antioxidant defences and lipid peroxidation in plasma exposed to the gas phase of CS

Fresh plasma (40 ml) in a 500 ml filter flask was exposed to the gas phase of CS as described in the Materials and methods section. Levels of endogenous plasma antioxidants are given as percentages of their initial concentrations, which were as follows: ascorbic acid (○), 45 μM; protein thiols (+), 421 μM; albumin-bound bilirubin (△), 13.9 μM; uric acid (×), 373 μM; α-tocopherol (□), 38 μM. The concentrations of hydroperoxides of plasma phospholipids (■), triacylglycerols (●) and cholesterol esters (▲) are also shown. One experiment representative of three is shown.

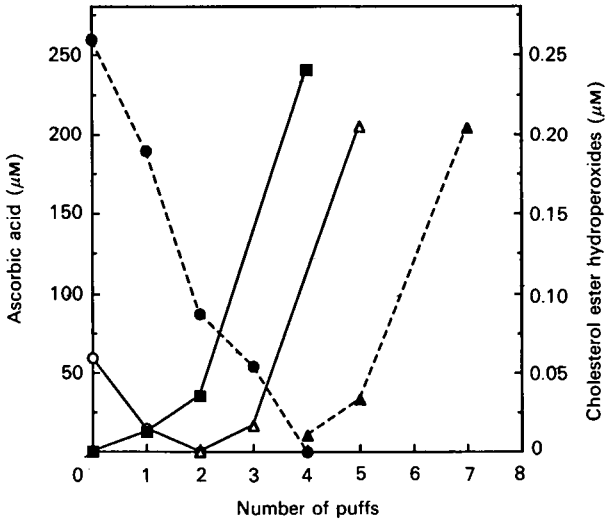

Fig. 3. Protection by ascorbic acid of plasma lipids against detectable peroxidative damage induced by the gas phase of CS

Plasma was treated with or without 0.5 units of ascorbate oxidase/ml for 15 min at 25 °C, or 200 μM-ascorbic acid was added to untreated plasma. Treatment with ascorbate oxidase led to oxidation of > 99% of endogenous ascorbic acid. Each plasma preparation (20 ml in a 250 ml filter flask) was exposed to the CS gas phase as described in the Materials and methods section. Lipid peroxidation was assessed by measuring the formation of cholesterol ester hydroperoxides (CE-OOH). ■, CE-OOH formation in plasma treated with ascorbate oxidase; ○, ascorbic acid consumption in control plasma; △, CE-OOH formation in control plasma; ●, ascorbic acid consumption in plasma to which ascorbic acid had been added; ▲, CE-OOH formation in plasma to which ascorbic acid had been added. One experiment representative of three is shown.

hydroperoxides of plasma phospholipids, cholesterol esters and triacylglycerols. These results demonstrate that oxidants present in the gas phase of CS are capable of inducing detectable lipid peroxidation in plasma, once ascorbic acid has been consumed completely.

In control experiments, plasma was exposed to air instead of the gas phase of CS. Ascorbic acid was oxidized at a slow and steady rate (30% and 55% oxidized after three and six sham air exposures over 1 and 2 h respectively). The other plasma antioxidants were not oxidized, nor were detectable amounts of lipid hydroperoxides formed. When endogenous ascorbic acid was first oxidized by treatment of plasma with 0.5 units of ascorbate oxidase/ml for 15 min, subsequent exposures to air did not lead to formation of detectable amounts of lipid hydroperoxides after three exposures, and after six exposures only 20 nM-cholesterol ester hydroperoxides could be detected, but no other lipid hydroperoxides. These findings indicate that the lipids in plasma undergo slow autoxidation in air once ascorbic acid has been completely oxidized.

To further investigate the antioxidant efficacy of ascorbic acid, plasma was either depleted of endogenous ascorbic acid or supplemented with exogenous ascorbic acid prior to CS exposure. As shown in Fig. 3, in plasma devoid of ascorbic acid due to treatment with ascorbate oxidase, lipid peroxidation was initiated immediately upon exposure of the plasma to the gas phase of CS, whereas in plasma containing added ascorbic acid, initiation of detectable lipid peroxidation was delayed significantly. These data demonstrate that ascorbic acid is the only endogenous antioxidant in plasma that can completely prevent detectable peroxidative damage to lipids.

Peroxidation of plasma lipids by gas-phase CS was accompanied by subtle changes in the electrophoretic mobility of the major lipoprotein classes (Figs. 4*a* and 4*b*). In each of five subjects studied, exposure of plasma to the CS gas phase was associated with a slight but consistent increase in mobility of the particles associated with the β-migrating band. α-Migrating

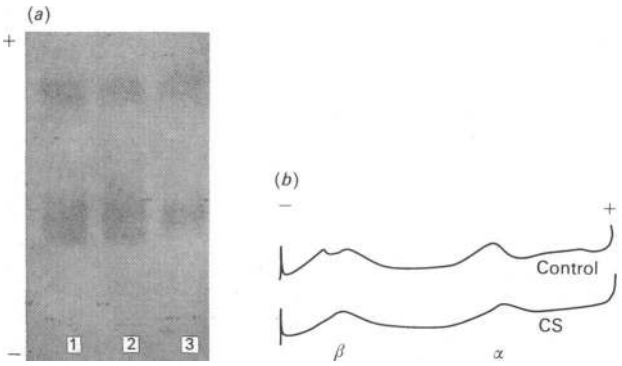

Fig. 4. Effects of CS on lipoproteins

(*a*) Representative agarose gel electrophoresis patterns of plasma incubated for 2 h at 4 °C in the absence of gas phase CS (lane 1), incubated for 2 h at 37 °C in the absence of gas phase CS (lane 2) and incubated for 2 h at 37 °C with six consecutive puffs of gas phase CS (lane 3). (*b*) Densitometric scans of samples incubated at 37 °C in the absence (control) and presence of CS gas phase. Shifts in migration pattern are more readily discerned in the scans. The β-, preβ-, and α-migrating bands (LDL, IDL/VLDL and HDL respectively) are indicated. Compared with the control samples, the β band migrates somewhat more negatively in the sample exposed to CS gas phase. The α-migrating band becomes broadened and more diffuse after exposure to the gas phase.

particles also appeared to have undergone slight changes after CS exposure where, compared with the control sample, the α band was more diffuse and shifted slightly toward the anode.

The increased migration of β lipoproteins after exposure of plasma to gas-phase CS, although slight, was reminiscent of

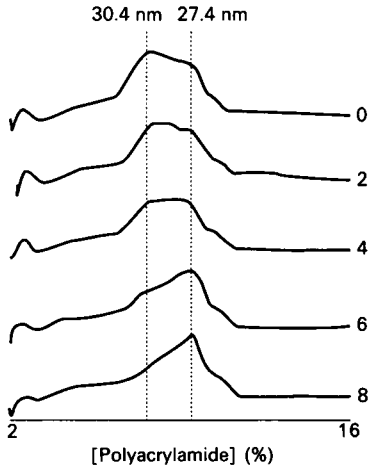

Fig. 5. Non-denaturing polyacrylamide gradient gel (2–16%) scans of plasma exposed to increasing amounts of CS gas phase

The number of puffs is shown on the right. In the control sample maintained at 37 °C with air, there is a pronounced peak at 30.4 nm which corresponds in size to IDL and small VLDL particles; a second component, seen as a shoulder, bands at approximately 27 nm and corresponds with LDL particles. With increasing exposure to the gas phase, there is a decrease in staining intensity of 30.4 nm material and a relative increase in that of 27.4 nm particles. Gels were stained with Oil Red O before scanning.

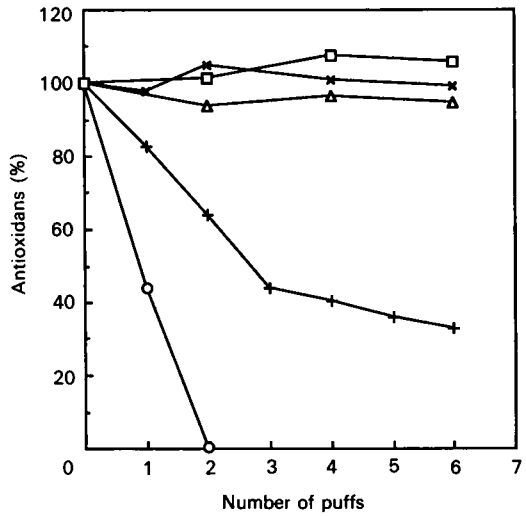

Fig. 6. Antioxidant defences and lipid peroxidation in plasma exposed to whole CS

Fresh plasma (40 ml) in a 500 ml filter flask was exposed to whole CS as described in the Materials and methods section. Levels of endogenous plasma antioxidants are given as percentages of their initial concentrations, which were as follows: ascorbic acid (○), 93 μM; protein thiols (+), 472 μM; albumin-bound bilirubin (△), 7.1 μM; uric acid (×), 285 μM; α-tocopherol (□), 32 μM. No lipid hydroperoxides could be detected (< 10 nM). One experiment representative of three is shown.

changes noted by others following oxidation of isolated LDL by copper [30,31]. It has also been previously reported that LDLs oxidized by incubation with endothelial cells become smaller and more dense as a consequence of peroxidation [32]. In order to determine whether gas-phase CS was also capable of altering the size distribution of LDL, plasma samples were analysed by non-denaturing gradient gel electrophoresis (Fig. 5). In the four cases examined, after exposure to six to eight puffs of filtered CS, there was a noticeable decrease in the intensity of Oil Red O-stained material around 30.4 nm, which is the banding region for intermediate-density lipoprotein (IDL) and small very-low-density lipoprotein (VLDL) [33]. Particles in the LDL size range (Fig. 5) showed little or no change in peak position; mean particle diameters were 27.5±0.4 nm and 27.5±0.3 nm ($n = 4$) for control and CS-exposed samples respectively. The decrease in IDL/small VLDL staining intensity was dependent on the extent of exposure to the CS gas phase, as suggested by the progressive decrease of the 30.4 nm component as a function of increased number of puffs. In two of the four experiments examined by non-denaturing gradient gel electrophoresis, it appeared that the decrease in IDL/small VLDL staining intensity was paralleled by a small increase in that of LDL; there was no apparent change in LDL in the other two experiments. Exposure of plasma to gas-phase CS did not result in fragmentation of LDL apo B protein, as determined by SDS/PAGE, nor was size or morphology altered as determined by electron microscopy (results not shown).

When plasma was exposed to whole CS, ascorbic acid and protein thiols became oxidized, whereas uric acid, albumin-bound bilirubin and α-tocopherol were not consumed (Fig. 6). In marked contrast to exposure of plasma to the gas phase CS, exposure to whole CS did not induce detectable lipid peroxidation or cause bilirubin oxidation (Fig. 6), nor did it change the electrophoretic mobility of LDL (results not shown). These findings suggest that formation of lipid hydroperoxides in plasma is related to changes in electrophoretic mobility of LDL contained in it; furthermore, they also suggest that the particulate matter present in whole CS possesses the ability to inhibit lipid peroxidation, perhaps because of the wide range of phenolic compounds present [4,34].

DISCUSSION

The chemical composition of CS is complex, with many free radical species, aldehydes, peroxides, epoxides, nitrogen oxides, peroxyl radicals and other pro-oxidants being present [4,34]. There is growing evidence that these oxidant species may contribute to the disease processes associated with smoking [35]. Thus reactive CS oxidant species have been implicated in emphysema [5], atherogenesis [15] and cancer [36,37].

The present studies were undertaken to investigate the oxidative effects of filtered and unfiltered CS and the protective effects of natural antioxidants in human extracellular fluids. The findings of this paper, obtained with human blood plasma, might also be relevant to oxidation and antioxidant defence reactions in other extracellular fluids, particularly the respiratory tract epithelial cell lining fluid(s), which *in vivo* are directly exposed to CS.

Our experiments have shown that exposure of plasma to the gas phase of CS causes rapid depletion of ascorbic acid followed by oxidative damage to lipids. Enzymic oxidation of ascorbic acid in plasma before exposure to CS gas phase led to immediate peroxidation of lipids, whereas supplementation of plasma with ascorbic acid delayed the onset of lipid peroxidation. Thus ascorbic acid appears to be the most important plasma antioxidant protecting lipids against CS-induced oxidation. In previous work we have shown that exposure of plasma to a chemically defined source of aqueous peroxyl radicals or activated neutrophilic phagocytes also leads to preferential depletion of ascorbic acid, and that in these systems, too, ascorbic acid is the only natural antioxidant that can protect the lipoprotein lipids from detectable peroxidative damage [22–24]. Peroxyl radicals are one of the most abundant types of free radicals present in the gas phase of CS [4,34]. As CS is also known to activate pulmonary macrophages and to activate and recruit monocytes and neutro-

phils to the lung [5–10,38], oxidants released from these phagocytes can be expected to play an additional role in CS-induced oxidative stress *in vivo*.

Isolated lipoproteins, particularly LDL, are prone to oxidation when separated from their normal plasma environment, especially in the presence of iron and copper ions [13,31,39,40]. However, in plasma it would be expected that physiological antioxidants would be able to protect the lipoproteins against oxidative damage [22–24]. In the present studies we show that, in whole plasma, detectable lipid peroxidation is initiated and lipoproteins are modified only after ascorbic acid oxidation is complete. Thus it appears that significant oxidative modification of LDL *in vivo* does not occur unless ascorbic acid has been depleted completely. Since such conditions would be preceded by overt symptoms of scurvy, it has been suggested that oxidation of LDL occurs in the extracellular fluid of the subendothelial space in microenvironments to which ascorbic acid has no access [14]. It is unlikely, however, that ascorbic acid has no access to a site in the subendothelial space which is accessible to much larger lipoproteins. A more likely scenario is that ascorbic acid is depleted locally in such microenvironments, for example due to activation of nearby monocytes/macrophages, and that subsequently LDL present at the same site becomes oxidatively modified.

Following ascorbic acid depletion, lipid hydroperoxides are formed and LDL becomes slightly more negatively charged in plasma exposed to gas-phase CS. However, we found no evidence of apo B fragmentation. This is in contrast to reports in which isolated LDL exposed to copper ions or to CS extract showed extensive fragmentation of apo B [30,31]. High levels of lipid peroxidation without concomitant damage to apo B have also recently been reported for isolated LDL exposed to u.v. irradiation [41]. The presence of other plasma proteins capable of reacting with lipid hydroperoxides or their aldehydic breakdown products [42] and the short incubation times (2–3 h) used in the present study, as compared with studies on isolated LDL, may help to explain the lack of apo B fragmentation.

The most obvious change in plasma lipoproteins after exposure to CS gas phase was the decrease in IDL/small VLDL lipid-staining material, as judged by non-denaturing gradient gel electrophoresis. This, coupled with the fact that there was no decrease in LDL staining, suggests that in whole plasma the lipid-rich IDL/small VLDL is more vulnerable to oxidation than other lipoproteins. It is possible that the oxidized lipid from IDL/small VLDL transfers to LDL, a hypothesis that would account for the increase in LDL staining in two out of four cases. Alternatively, oxidized lipids may have transferred to HDL, thus accounting for the alteration in HDL charge noted on agarose electrophoresis. It has recently been demonstrated that HDL can decrease oxidative damage to LDL protein during lipid peroxidation of LDL exposed to endothelial cells in the presence of copper ions. The suggested role of HDL in this process was that lipid peroxidation products of LDL, particularly oxidized phospholipids, were exchanged with unoxidized phospholipid from HDL [43].

The decrease in Oil Red O-staining material in the region corresponding to IDL/small VLDL upon exposure to gas-phase CS is an unexpected finding. Although speculative, there are several possible mechanisms which could account for this decrease in mass, including the following. (1) Lipoprotein lipase activity in plasma may have been stimulated by some constituent(s) in CS, thus reducing the triacylglycerol core of these particles. (2) A phospholipase A_2-like enzyme [44] may have been activated and preferentially hydrolysed phospholipids on the larger, less dense, apo B-containing particles.

Another interesting observation is that whole CS did not induce peroxidation of plasma lipids, although ascorbic acid, and to some extent protein thiols, became oxidized. In this respect, it is noteworthy that gas-phase CS represents a stronger oxidizing species than whole CS, possibly because of the antioxidant capabilities of some of the melanin-like phenolic pigments present in the particular fraction [4,34].

In summary, we have demonstrated that CS oxidants are capable of oxidizing naturally occurring plasma antioxidants, protein thiol groups and plasma lipids. The results support the concept that ascorbic acid is an important antioxidant in protecting extracellular lipid from oxidant stress [22–24,40,45,46], suggest that acute CS exposure has the potential to modify lipoproteins so that they become atherogenic, and strengthen the argument that augmented vitamin C intake is of benefit to cigarette smokers [46–48].

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