Modification of plasma proteins by cigarette smoke as measured by protein carbonyl formation

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Exposure of human plasma to gas-phase (but not to whole) cigarette smoke (CS) produces oxidative damage to lipids [Frei, Forte, Ames & Cross (1991) *Biochem. J.* 277, 133–138], which is prevented by ascorbic acid. The ability of CS to induce protein damage was measured by the carbonyl assay and by loss of enzyme activity and protein -SH groups. Both whole and gas-phase CS caused formation of carbonyls in human plasma, which was partially inhibited by GSH but not by ascorbic acid or metal-ion-chelating agents. Isolated albumin exposed to CS showed much faster carbonyl formation (per unit protein) than did whole plasma; damage to isolated albumin was partially prevented by chelating agents. Isolated creatine kinase (CK) lost activity upon exposure to CS much faster than did CK in plasma. Direct addition to plasma of mixtures of some or all of the aldehydes reported to be present in CS caused protein carbonyl formation and inactivation of CK, but neither occurred to the extent produced by CS exposure.

INTRODUCTION

Cigarette smoke (CS) is rich in free radicals [1], and it can also accelerate the production of reactive oxygen species by recruiting and activating phagocytes in the lung [2,3]. Thus it is widely believed that at least some of the deleterious effects of CS involve oxidative damage [1–3].

The first biological fluids that come into contact with inhaled CS are the respiratory tract lining fluids (RTLFs). Some information is available about the antioxidant ability of these fluids [4–7], but the problems of sampling them (by respiratory tract lavage) have greatly hindered elucidation of their precise chemical composition, since lavage produces considerable and variable dilution of RTLFs, and some constituents may be oxidized during the lavage and analytical preparation procedures [8,9]. Ascorbic acid, GSH and uric acid are all thought to be important antioxidants in the RTLFs [4–7].

By contrast, the antioxidant defences of human plasma have been well characterized [10]. For example, when plasma is exposed to the gas phase of CS, ascorbic acid appears to be the 'first line of defence' against lipid damage, and its disappearance is accompanied by the onset of lipid peroxidation [11]. There is little oxidation of uric acid [11]. Indeed, most studies of antioxidants in human body fluids have focused on their ability to protect lipids against peroxidation [10,11]. However, reactive oxygen species can damage many other molecules, including proteins [12,13] and DNA (reviewed in [14]). Indeed, damage to proteins and DNA may often be more important than damage to lipids in oxidative stress situations *in vivo* [12–16]. It has already been shown that CS causes oxidation of protein -SH groups in plasma [11].

Attack by reactive oxygen species upon proteins can damage several amino acid residues, including histidine, tryptophan, cysteine, proline, tyrosine, methionine, arginine and lysine [12,17]. Oxidative damage to several of these amino acid residues

and/or to the peptide backbone of proteins can generate carbonyl products [12,17]. Indeed, measurement of 'protein carbonyls' has been used as a sensitive assay for oxidative damage to proteins [12,15,17], partly because it measures several different consequences of oxidative damage. However, reaction of some unsaturated hydroxyaldehydes with proteins has been suggested to form products that are also detectable in the carbonyl assay [17,18]. In the present paper, we have used the carbonyl assay to measure damage to isolated proteins, and proteins in human plasma, exposed to CS. CS has high levels of both free radicals [1] and aldehydes, especially acetaldehyde (ethanal), propanal and acrolein, but also butanal, isovaleraldehyde, formaldehyde and crotonaldehyde [19,20]. The protective effects of selected antioxidants were also studied.

MATERIALS AND METHODS

Blood collection

Blood from seven normolipidaemic male volunteers (age range 30–60 years) was drawn into heparinized vacutainers and centrifuged immediately at 1000 *g* and 4 °C for 10 min to obtain plasma, which was used immediately for the experiments described. In some experiments, serum was obtained by using non-heparinized containers and allowing blood to clot at room temperature.

Cigarettes

The cigarettes used in this study were University of Kentucky 2R1 standard cigarettes containing 23 mg of tar and 2.2 mg of nicotine per cigarette (according to the Federal Tobacco Council). Full details of their composition are given in [19,20].

Exposure of plasma to CS

This was carried out using a filter flask/vacuum system as

Abbreviations used: CS, cigarette smoke; RTLFs, respiratory tract lining fluids; DNPH, dinitrophenylhydrazine; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); CK, creatine kinase.

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described in [11]. Control plasma samples were incubated in similar flasks at 37 °C with 'puffs' of room air instead of CS.

Biochemical assays

Carbonyls were determined by a modification of the procedure described in [21], using dinitrophenylhydrazine (DNPH) dissolved in HCl, accompanied by blanks in HCl alone. After the DNPH reaction, proteins were precipitated with an equal volume of 20% (w/v) trichloroacetic acid and the pellets were washed once with 4 ml of 10% (w/v) trichloroacetic acid and three times with 4 ml of an ethanol/ethyl acetate mixture (1:1). Washings were achieved by mechanical disruption of the pellets in the washing solution using a small spatula, and re-pelleting by centrifugation at 6000 *g* for 5 min. Finally, the precipitates were dissolved in 6 M-guanidine-HCl solution and the absorbance peak at 320–400 nm was determined by spectral scanning. Protein contents were determined on the HCl blank pellets using a BSA standard curve in guanidine-HCl and reading the absorbance at 280 nm.

Ascorbic acid was measured by h.p.l.c. with electrochemical detection [22].

Protein thiols were measured using 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB). Absorbances were measured at 412 nm against blank samples without DTNB and also corrected for a no-sample blank [23]. The former control is important, because exposure of plasma to CS can cause development of absorbance at 412 nm. Absorbance readings were made 2 min after adding DTNB to test samples.

GSH and GSSG in plasma were measured by an enzyme recycling assay [24]. Briefly, GSH was determined by mixing equal parts (200 μ l) of plasma with 10 mM-DTNB solution. The resulting GSSG was measured spectrophotometrically by the rise in A_{412} after addition of glutathione reductase. The amount of GSSG in the sample was determined by removing GSH from the sample (200 μ l) with an equal volume of 10 mM-*N*-ethylmaleimide and then assaying for remaining GSSG.

Creatine kinase (CK) (ATP:creatine phosphotransferase; EC 2.7.3.2) was measured by a colorimetric assay based on the reaction of phosphocreatine with ADP to form creatine, measured by formation of a chromogen in the presence of α -naphthol and diacetyl (Sigma kit no. 520).

Lactate dehydrogenase, aspartate aminotransferase and γ -glutamyl transferase were measured by standard autoanalyser techniques (Beckman Synchron CX4).

Reagents

All reagents were of the highest quality available from Sigma Chemical Co., except that some samples of highly purified human albumin (certified for intravenous administration) were kindly donated by Cutter Laboratories, Berkeley, CA, U.S.A. CK was the rabbit muscle enzyme, type C3755.

RESULTS

Damage to proteins in human plasma by exposure to CS

Freshly prepared human plasma from seven different volunteers was exposed to CS from University of Kentucky 2R1 standard cigarettes. To obtain gas-phase CS, a Cambridge filter system capable of removing particles $\geq 0.01 \mu$ m in diameter was used to filter the CS [11]. Some volunteers provided plasma on several different occasions.

Fresh human plasma showed only low levels of protein carbonyls (0.72 ± 0.12 nmol/mg of protein; $n = 15$). Serum contained carbonyl levels in the same range. Carbonyl levels did not increase when plasma was incubated at 37 °C under air (tested up to 6 h). However, exposure of plasma to increasing numbers of

puffs of gas-phase CS produced a steady increase in protein carbonyls (Fig. 1). CS exposure also produced oxidation of -SH groups in plasma, as expected from previous studies [11], but there was little oxidation of uric acid. As expected [11], plasma -SH groups decreased by 30–40% after 3 h. Addition of desferrioxamine, an inhibitor of iron-ion-dependent free radical reactions [25], to a final concentration of 1 mM had no significant effect upon carbonyl formation (Fig. 1), nor did including both desferrioxamine (1 mM) and 1 mM-EDTA (three experiments). Ascorbic acid addition (final concentration 400 μ M–1 mM; four experiments; normal plasma concentration 40–120 μ M) exerted no significant protective effect against protein carbonyl formation (e.g. Fig. 1). Addition of 45 mM-glucose (in addition to normal plasma glucose of about 4.5 mM) did not inhibit carbonyl formation (Fig. 1). Indeed, the tendency was to increase carbonyls slightly, but this was not statistically significant ($P > 0.1$, $n = 3$). GSH (1 mM) partially decreased carbonyl formation (Fig. 1), but the combination of GSH and ascorbate did not produce an additive protective effect. As expected, both added and endogenous GSH and ascorbic acid were oxidized in plasma during CS exposure. However, analysis showed that some of the added GSH always persisted for at least 2 h, and some of the added ascorbate for at least 3 h. Hence the incomplete protection by GSH and the lack of protection by ascorbate cannot be attributed to their complete oxidation by CS.

Exposure of plasma to whole CS, rather than gas-phase CS, does not cause lipid hydroperoxide formation [11]. By contrast, both whole and gas-phase CS were equally damaging to proteins, as measured by carbonyl formation (Table 1). Addition of desferrioxamine or EDTA (final concentrations 1 mM) provided no protection for plasma proteins against damage by whole CS (e.g. Table 1).

Damage to individual proteins by CS exposure

Plasma contains a vast range of different proteins, but a major one is albumin. This protein is known to be susceptible to free radical damage [13,26–29]. Isolated human plasma albumin from two different commercial sources was found to contain higher concentrations of protein carbonyls per unit protein (range

Fig. 1. Effect of gas-phase (filtered) CS on the accumulation of plasma protein carbonyls in the absence or presence of various antioxidants

Final concentrations of additives were: desferrioxamine (1 mM) ascorbate (1 mM), glucose (~50 mM), GSH (1 mM). Results are means $\pm$ S.D., $n = 15$ (air or CS alone) or $n = 3$ (plus GSH, desferrioxamine, ascorbate or glucose).

3–5 nmol/mg of protein) than does freshly prepared human plasma or serum (<1.0 nmol/mg of protein). Exposure of albumin to gas-phase or whole CS produced a rapid increase in carbonyls (Fig. 2). Albumin carbonyl formation was partially inhibited by GSH: added GSH was oxidized by CS, but analysis showed that about one-third of the added GSH was still present at 3 h. Unlike the case of plasma, albumin carbonyl formation was partially inhibited by addition of desferrioxamine (Fig. 2). Addition of both desferrioxamine (1 mM) and EDTA (1 mM) did not further increase the protective effect.

Several enzymes were assayed in CS-exposed plasma. Exposure to up to nine puffs of gas-phase CS over a 3 h period at 37 °C produced no measurable change in the activities of lactate dehydrogenase, aspartate transaminase or γ -glutamyl transferase. By contrast, there was a significant decrease in CK activity (Table 2). Purified commercial CK exposed to gas-phase CS was much more rapidly inactivated than was the CK activity in plasma (Table 2). Exposure of isolated CK to gas-phase CS caused a loss of protein thiol groups and a rise in protein carbonyls (Table 3). The commercial protein again contained more carbonyls (per unit protein) than does freshly prepared human plasma (Table 3). Comparison of Tables 2 and 3 shows that exposure of CK to CS causes a rapid loss of enzyme activity and -SH groups, but significant carbonyl formation was not seen until after 2 h of exposure. This suggests that loss of -SH may be related to loss of enzyme activity, but that carbonyl formation does not cause the loss of CK activity.

Effect of aldehydes on plasma carbonyls

It has been suggested that binding of certain unsaturated hydroxyaldehydes to proteins might produce products reactive in the carbonyl assay [17,18]. CS is rich in aldehydes (about

Fig. 2. Effect of gas-phase CS on human serum albumin in the absence or presence of desferrioxamine or GSH

Antioxidants were present at 1 mM final concentrations. Albumin was dissolved in 100 mM-KH₂PO₄/K₂HPO₄ buffer, pH 7.4 at 70 mg/ml (the approximate total protein concentration in human plasma). Results are means $\pm$ S.D.; *n* = 3. No increase in carbonyls was observed in albumin solutions incubated at 37 °C in the absence of CS.

Table 3. Effect of CS on free thiol groups and carbonyl content of rabbit muscle CK

Results are means $\pm$ S.D.; *n* values are given in parentheses. There was no significant change in carbonyls in enzyme incubated under air, but a slow loss of -SH groups was observed (10–15% after 3 h); this has been corrected for in the data presented.

Exposure time to CS (h)	-SH groups (% of starting value)	Carbonyl content (nmol/mg of protein)
0	100	3.0 $\pm$ 0.4 (4)
1	40 $\pm$ 8 (2)	3.1 $\pm$ 0.6 (3)
2	20 $\pm$ 5 (3)	5.5 $\pm$ 1.2 (3)
3	1 $\pm$ 1 (3)	13.0 $\pm$ 3.3 (3)

Table 4. Effect of aldehydes on carbonyls in human plasma

Aliquots of freshly prepared plasma were exposed to gas-phase CS (1 puff every 20 min; 9 puffs in all [11]) or treated every 20 min with 20 μ l of a freshly prepared aqueous solution containing the aldehydes present in CS. Nine additions of 20 μ l gave a total addition of 1000 μ g of acetaldehyde, 50 μ g of formaldehyde, 39 μ g of propanal, 10 μ g of butyraldehyde, 10 μ g of isovaleraldehyde, 45 μ g of acrolein and 15 μ g of crotonaldehyde. Samples were analysed every hour for protein carbonyls, as described in the Materials and methods section. Results are means $\pm$ S.D.; *n* = 4 except where indicated in parentheses.

Time (h)	Protein carbonyls (nmol/mg of protein)		
	Plasma exposed to air at 37 °C	Plasma exposed to CS	Plasma treated with aldehydes
0	0.72 $\pm$ 0.12 (15)	—	—
1	—	2.11 $\pm$ 0.20	1.07 $\pm$ 0.18 (3)
2	—	3.80 $\pm$ 0.45	1.15 $\pm$ 0.18 (3)
3	0.72 $\pm$ 0.12 (15)	4.88 $\pm$ 0.61	2.28 $\pm$ 0.14 (3)

Table 1. Effect of whole CS on plasma protein oxidation as determined by protein carbonyls

Results are means $\pm$ S.D.; *n* values are given in parentheses. There was no significant difference between results in the absence or the presence of desferrioxamine.

Time (h)	Protein carbonyls (nmol/mg of protein)	
	Whole CS	Whole CS + desferrioxamine
0	0.72 $\pm$ 0.12 (15)	0.73 $\pm$ 0.14 (4)
1	3.01 $\pm$ 0.18 (3)	2.97 $\pm$ 0.18 (3)
2	3.72 $\pm$ 0.25 (3)	3.55 $\pm$ 0.36 (3)
3	6.11 $\pm$ 1.05 (3)	6.21 $\pm$ 1.12 (3)

Table 2. Activity of human plasma CK and purified CK after exposure to gas-phase CS for up to 3 h

Rabbit muscle CK was dissolved in 100 mM-KH₂PO₄/KOH buffer, pH 7.4, at 5 mg/ml. Results are means $\pm$ S.D., *n* = 5. There was no loss of CK activity when plasma or purified CK were incubated at 37 °C in the absence of CS.

Source of CK activity	Activity after exposure to CS (% of initial activity)		
	1 h	2 h	3 h
Human plasma	80 $\pm$ 4	76 $\pm$ 4	60 $\pm$ 3
Rabbit muscle enzyme	40 $\pm$ 5	30 $\pm$ 6	18 $\pm$ 6

Fig. 3. Protein carbonyl formation on addition of aldehydes to plasma

One UK research-grade cigarette contains approx. 1262 μg of total aldehydes. A mixture of acetaldehyde, acrolein and propanal in the relative amounts found per cigarette (849:45:128, by weight) equivalent to 1262 μg of aldehyde was divided into nine aliquots which were added to fresh human plasma at time zero and every 20 min thereafter for 3 h in total, to mimic the puffing with CS. Carbonyls were measured in plasma aliquots drawn at 0, 1, 2 and 3 h. Results are means $\pm$ S.D.; $n = 3$. Where indicated, ascorbate and GSH were added to give final concentrations of 1 mM.

1260 μg /cigarette; [19,20]). The major ones present [19,20] in CS from the Kentucky research-grade cigarettes used here are acetaldehyde (849–1000 μg /cigarette), propanal (40–128 μg /cigarette), acrolein (45 μg /cigarette), crotonaldehyde (15 μg /cigarette), formaldehyde (50 μg /cigarette), butyraldehyde (10 μg /cigarette) and isovaleraldehyde (10 μg /cigarette). The effects of these aldehydes upon human plasma were studied. When a mixture of these aldehydes in the proportions reported to be present in CS was added to plasma and incubated at 37 °C, some increase in protein carbonyls was detected (Table 4). It should be noted that the protein precipitation and thorough washing procedures used preclude any direct reactivity of aldehydes in the carbonyl assay. The aldehydes were added in aliquots at 20 min intervals, to mimic the CS exposure experiments (9 puffs consume $\geq 90\%$ or more of one cigarette). The effect of aldehydes on the CK activity of plasma was also examined. The mixture of aldehydes produced only $51 \pm 12\%$ (mean $\pm$ S.D., $n = 5$) of the loss of CK activity produced by CS at 1, 2 or 3 h in parallel exposures on the same plasma samples. The aldehydes produced no measurable loss of any of the other plasma enzyme activities measured in this study.

Fig. 3 shows a similar experiment, but using only three of the major aldehydes found in CS (acetaldehyde, acrolein and propanal). GSH protected the plasma against aldehyde-induced protein carbonyl formation (Fig. 3), but ascorbic acid had little effect.

DISCUSSION

Exposure of plasma to CS causes oxidative damage to proteins, as measured by the loss of -SH groups and by the well-established method of carbonyl formation [12,15,17]. In the case of CK, the carbonyl formation was not related to loss of enzyme activity; only when the amino acid residues oxidized are essential for catalytic activity (as may be the case for CK -SH groups) will such a relationship be expected.

Commercial human plasma albumin contained higher baseline levels of protein carbonyls, and developed carbonyls at a higher rate (per unit protein) upon CS exposure, than did plasma proteins. This suggests that some other agents in the plasma are able to protect albumin. Indeed, we found that GSH protected both plasma proteins and isolated albumin against carbonyl formation. Although human plasma GSH concentrations are only 1–2 μM , some RTLFs are reported to contain much higher levels [4,7], and so GSH might be a significant protector against damage by CS and/or inhaled aldehydes in the bronchiolar-alveolar regions of the respiratory tract. CS-induced damage to isolated albumin was partially inhibited by chelating agents, probably because commercial albumin is contaminated with transition metal ions such as iron and copper. These could react with peroxides in CS to form protein-damaging radicals such as hydroxyl ($\cdot\text{OH}$) [13,27]. By contrast, freshly prepared human plasma does not contain any free iron or copper ions [10,30], hence explaining the lack of protection by chelating agents. These data illustrate one of the potential problems in extrapolating data on free radical damage to isolated molecules (such as proteins or lipids) to the situation *in vivo*.

Both whole and gas-phase CS were equally damaging to plasma proteins. By contrast, although gas-phase CS produces lipid oxidation in plasma, whole CS did not cause any detectable lipid hydroperoxide formation [11]. The particulate phase of CS is rich in phenolic compounds which may exert chain-breaking antioxidant effects with lipids, while not protecting (or even accelerating damage to) other biological molecules [31,32]. CS also inactivated plasma CK, although the rate of inactivation of the isolated enzyme was much greater than that of the endogenous plasma enzyme, perhaps because other plasma constituents (such as albumin) intercept many of the damaging species and so protect the plasma CK.

What species in CS cause protein carbonyl formation? One possibility is protein-damaging radicals, such as $\cdot\text{OH}$, peroxy and alkoxyl [13]. However, plasma contains many powerful scavengers of $\cdot\text{OH}$ [32], and the $\cdot\text{OH}$ scavenger glucose (at 50 mM final concentration) failed to protect proteins. Neither EDTA nor desferrioxamine were protective against protein carbonyl formation in plasma; these chelators would be expected to inhibit iron (desferrioxamine) and copper (EDTA)-mediated generation of $\cdot\text{OH}$ from H_2O_2 . Both peroxy and alkoxyl radicals react very quickly with ascorbic acid [32], which is presumably why even low concentrations of ascorbic acid protect plasma lipids against CS-induced peroxidation [11]. The failure of ascorbate to inhibit protein carbonyl formation suggests that peroxy and alkoxyl radicals are not involved in the latter process.

Our data show that addition to plasma of a mixture of the aldehydes found in CS produces products detectable in the carbonyl assay and causes some inactivation of CK but not of the other plasma enzymes. GSH inhibits this carbonyl formation but ascorbate has little effect, results similar to those in CS-exposed plasma. We conclude that at least some of the carbonyl formation and CK inactivation caused by CS is due to aldehydes. However, addition of aldehydes in amounts equivalent to those in CS did not result in as much protein carbonyl formation or CK inactivation as did exposure to CS. It could be that aldehydes account for part, but not all, of the effects of CS on plasma, and that CS produces protein damage by more than one mechanism. It might also be that the mixture of aldehydes we used does not entirely mimic those produced in CS.

Why should aldehydes generate carbonyl products? Acrolein reacts very quickly with -SH groups, initially forming a 1:1 adduct which contains a carbonyl group [33]. This may account for the formation of carbonyls after addition of acrolein to

plasma. By contrast, the exact chemistry by which acetaldehyde and the other aldehydes we tested could react to form protein carbonyls is uncertain. They would be expected to combine with -SH or -NH₂ groups to give adducts without a carbonyl group. In the carbonyl assay, protein is incubated with DNPH and then precipitated and thoroughly washed, so that any aldehydes displaced from the protein by DNPH are removed. Whatever the mechanism of carbonyl formation, our results show that caution should be used in interpreting the results of protein carbonyl assays upon systems containing large quantities of aldehydes.

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