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Oxidative damage to plasma constituents by ozone

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The reaction of ozone (O_3) with human blood plasma was studied to help understand possible events that could occur in the respiratory tract. Uric acid (quantitatively the most important scavenger) and ascorbic acid were oxidized quickly, protein-SH groups were lost more slowly, and there was no loss of bilirubin or α-tocopherol. There was little formation of lipid hydroperoxides and no detectable formation of 4-hydroxynonenal, hexanal or nonanal, or changes in lipoprotein electrophoretic mobility. Uric acid in human upper airway secretions may play a significant role in removing inhaled O_3. Oxidative damage to lipids must not be assumed to be the key mechanism of respiratory tract O_3 toxicity.

Ozone; Uric acid; Oxidative damage; Protein damage; Lipid peroxidation

1. INTRODUCTION

Ozone (O_3) is an important toxic component of photochemical air pollution. It is believed that the powerful oxidizing ability of O_3 is responsible for its adverse biological effects [1–4]. O_3 can oxidize several biological molecules directly [1,4] and, in addition, it reacts slowly with water at physiological pH to yield highly reactive hydroxyl radicals [5]. Thus several biological antioxidants, especially α-tocopherol, have been hypothesized to exert protection against damage by O_3 in vivo [1,2] and it is widely believed that oxidative damage to lipids by O_3 is a major mechanism of its toxicity [1,2,6].

The first biological fluids that come into contact with inhaled O_3 are the respiratory tract lining fluids (RTLFs), which presumably serve to absorb and detoxify some of the inhaled O_3 so as to lower the amount that enters the more-vulnerable peripheral gas exchange regions of the lung [7,8]. Some information is available about the antioxidants of these fluids [8–13] but the problems of sampling them (by the techniques of respiratory tract lavage) have hindered elucidation of their precise comparative antioxidant capabilities, since lavage itself produces considerable and variable dilution of RTLFs and some of their constituents may be oxidized during the procedures [14,15].

By contrast, the antioxidant defences of human plasma have been well characterized (reviewed in [16]). When plasma is exposed to chemically produced peroxyl radicals, to cigarette smoke or to oxidants generated by activated neutrophils, ascorbic acid appears to be a 'first line of defence' and its disappearance is accompanied by the onset of peroxidation of plasma lipids [17–19]. By contrast, although uric acid (a suggested physiological antioxidant [20]) has been demonstrated to have antioxidant properties in vitro [3,20–22], it has not been found to date to play a major protective role in plasma exposed to oxidants [16]. We show here that uric acid is probably the most important scavenger of O_3 in human blood plasma, and we relate this observation to the presence of uric acid in upper RTLFs [8,23].

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2. MATERIALS AND METHODS

2.1. *Plasma O_3 exposure*

Blood was drawn from healthy adult male volunteers (age range 30–60) into heparinized tubes and centrifuged to obtain plasma. 5 ml aliquots were placed in Falcon dishes in a closed container at 37°C and exposed to a humidified constantly monitored level of 16 ppm O_3 in 5% CO_2/95% air as described in [24]. Control plasma was exposed to an identical gas-stream but without O_3. Measurement of the concentrations of plasma electrolytes (using a Beckman Synchron CX4 autoanalyzer) showed that, as expected [24], even 12 h of exposure caused no evaporation of water from the plasma, nor did the plasma pH change.

2.2. *Antioxidants*

Antioxidants and uric acid oxidation products were measured by specific HPLC-based methods, and protein thiols using Ellman's reagent [17–19,25].

2.3. *Measurement of aldehydes*

Samples were analyzed for *trans*-4-hydroxy-2-nonenal (HNE) by forming the pentafluorobenzyl oxime trimethylsilyl ether derivative and using GC/electron capture ionization mass spectrometry and selected ion monitoring, by modifications of the methods described in

[26,27]. Quantitative analysis was performed by using a deuterated HNE internal standard. HNE was synthesized as in [28] and its deuterated analogue as in [27]. Both were stored as solutions in dichloromethane at −80°C. Standard aqueous solutions of aldehydes were prepared by evaporating the solvent under reduced pressure and dissolving a weighed amount of the residue in a known volume of deionized water. Concentrations were further checked by measuring the absorbance of the aqueous solution at 223 nm. *o*-(2,3,4,5,6-Pentafluorobenzyl)-hydroxylamine hydrochloride (PFB·HCl) and *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide (MSTFA) were obtained from Aldrich Chemical Co. (Milwaukee, WI). Hexane (Optima grade) was from Fisher Chemical Co. (Fair Lawn, NJ).

Samples were extracted by a modification of the method in [27]. To a conical 15 ml centrifuge tube was added 250 μl of butylated hydroxytoluene solution (10 mg/ml in ethanol), 200 μl of 2 mM EDTA, pH 7.0, a volume of aqueous solution containing 10 ng of HNE-d_2 standard, and 200 μl of 0.05 M *o*-PFB·HCl in 0.1 M PIPES buffer, pH 7.0. The mixture was vortexed for 1 min and allowed to stand at room temperature for 5 min. The mixture was then extracted 3 times by adding 1 ml of hexane, vortexing, centrifuging, and removing the upper organic layer. The hexane extracts were combined, the solvent evaporated under a stream of N_2 and 50 μl of MSTFA added. Samples were analyzed by CC/electron capture ionization mass spectrometry using a VG Trio-2 GC/MS (VG Masslab, Altrincham, UK) instrument with a 15 m DB-5 capillary column (J&W Scientific, Folsom, CA) and splitless injection at an injector temperature of 250°C and He as carrier gas at a linear velocity of 35 cm/s. Oven temperature was programmed from 50°C to 250°C at 10°C/min. Electron capture ionization (negative ion) was performed using methane as a buffer gas at a pressure of 5×10^{-5} mbar measured at the diffusion pump manifold. The source temperature was held at 150°C. Selected ion monitoring of *m/z* 152 and 154 was performed, corresponding to loss of the pentafluorobenzyl and OTMS groups from the pentafluorobenzyl oxime derivatives of 4-HNE and its deuterated analogue. Separation of syn and anti oxime isomers was observed. A calibration curve was linear over 3 orders of magnitude ($r^2 = 0.998$). Concentration of HNE was calculated by measuring the 152/154 peak area ratio and comparing to the calibration curve, and expressed as ng HNE/ml. Similar measurements were made for hexanal and nonanal, again using deuterated standards.

2.4. *Lipid hydroperoxides*

These were separated by HPLC and quantitated by post-column derivatization with chemiluminescence detection [15–17].

3. RESULTS

3.1. *Depletion of antioxidants in O_3-exposed plasma*

Figure 1 shows a representative experiment revealing what happens to antioxidants when freshly-prepared human plasma is exposed to O_3. Sixteen ppm of O_3 was used to accelerate (for ease of measurement) the oxidative changes observed, since the surface/volume ratio in our experimental system is necessarily far lower than the enormous value found in the respiratory tract [29]. Similar results were obtained in 9 different experiments, utilizing plasma freshly obtained from 3 different donors at different times.

There was a rapid depletion of both uric acid and ascorbic acid: uric acid did not decline in the air-exposed controls and ascorbic acid remained constant (1 experiment) or fell by 8–19% of its initial value (8 experiments) after 4 or 6 h incubation. Protein-SH groups declined more slowly in O_3-exposed plasma. By contrast, there was no significant change in α-tocopherol

Figure

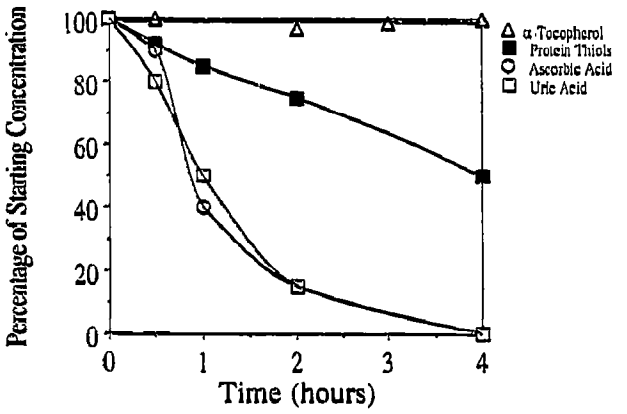

Fig. 1. Depletion of antioxidants and protein thiol groups in human plasma exposed to 16 ppm O_3. Results are expressed as % of antioxidant present (100% at zero time); some actual values are shown in Table 1. Assays were performed as described in [15–18]. Ascorbate results are corrected for the slow loss of ascorbate in an air-exposed control. The other antioxidants and protein thiols did not change in air-exposed controls.

(the major inhibitor of lipid peroxidation in human plasma) or of bilirubin (not shown). Addition of catalase (10^3 enzyme units/ml) to the plasma before O_3 exposure to remove any H_2O_2 generated did not alter the results.

The concentration of uric in human plasma is 5–10 times greater than that of assorbic acid [20]. Since both of them disappear at approximately equal percentage rates when plasma is exposed to O_3, it follows that more of the O_3 reacts with uric acid than with ascorbic acid (assuming that both molecules react with O_3 on a 1:1 molar basis). Thus, exposure of 9 plasma samples to 16 ppm O_3 for 30 min led to a mean loss of 23 μM ascorbic acid (range 5–33 μM), but 89 μM uric acid (range 62–116 μM). After 1 h exposure, a mean of 35 μM ascorbic acid (range 21–54 μM) was lost, but 170 μM (range 106–225 μM) uric acid. After 1 h, a mean of 75 μM (range 37–128 μM) protein thiols had been lost.

Oxidation of uric acid by reactive oxygen species generates several end-products, but the major one is usually allantoin [25]. This was also true for O_3. Thus, in a representative plasma sample, 2 h exposure to 16 ppm O_3 oxidized 295 μM uric acid, and 80 μM allantoin was formed. Several other (unidentified) products were detected by HPLC [25]. O_3 is reported to react with H_2O to form ·OH [5]. However, oxidation of 400 μM uric acid in phosphate-buffered saline by 16 ppm O_3 was not slowed by adding the ·OH scavenger mannitol, tested up to 500 mM. Hence, even in buffer solution, ·OH radicals appear not to contribute to the uric acid oxidation. They are even less likely to do so in plasma, which contains a vast range of compounds that scavenge ·OH [16,30]. Hence O_3 probably oxidizes uric acid and other plasma constituents directly.

The effects of adding extra ascorbic acid or uric acid to plasma, or of depleting plasma or uric acid by adding urate oxidase (1 unit/ml of Sigma uricase enzyme, added 10 min before O_3 exposure) were examined. HPLC confirmed that the uric acid had been oxidized. Table I summarizes the results of several experiments. In general, the greater the amount of ascorbic acid or uric acid present, the faster the rate at which they were oxidized. Adding extra ascorbate did not slow the loss of uric acid, nor did depletion of uric acid (or adding extra) alter the loss of ascorbic acid (Table I). Similarly, adding ascorbate or uric acid had only small effects on the loss of protein-SH groups (Table I).

3.2. *Attempts to measure lipid peroxidation*

Measurement of lipid hydroperoxides by a sensitive and specific assay [17–19] showed that only low concentrations were formed (always <1 μM, even after 6 h exposure to O_3 in 6 experiments). Even these low levels were only measurable after prolonged (>2 h) exposure to O_3.

It could be argued that low levels of lipid hydroperoxides are due to their breakdown to other products, such as the cytotoxic aldehyde hydroxynonenal [31]. However, direct measurement of this aldehyde by displacement from protein, derivatization and GC-MS found none detectable in 6 different samples exposed to O_3 for 2–6 h (limit of detection of the method used 0.01 ng/ml). The preparation procedure used detects aldehyde bound to protein amino groups, but not to -SH groups. However, this is allowed for by calibrating the system with deuterated 4-hydroxynonenal added directly to plasma (70–75% recovery). Another possibility is that O_3 fragments fatty acid side chains directly into aldehydes [32], such as hexanal and nonanal. However, analysis of the plasma samples for these two aldehydes failed to detect them. (Limit of detection 1 ng/ml.) Again, 70% detection of aldehyde standards added to plasma was achieved.

Plasma lipoproteins are an important target for oxidative lipid damage [31,33]. However, no shifts in electrophoretic mobility of the different plasma lipoprotein species [33] were observed by agarose gel electrophoresis [19] after exposing plasma to O_3 (tested up to 12 h) when compared with air-exposed controls.

4. DISCUSSION

Uric acid and ascorbic acid appear to be the major scavengers of O_3 in plasma, but adding more of them causes them to be oxidized faster. How can this be explained? The reaction of O_3 with body fluids is probably an example of *reactive absorption* [34,35], i.e. the more oxidizable material present in the fluid, the more O_3 is absorbed by the fluid to react with this material. When O_3 is inhaled, the antioxidants in the upper RTLFs should combine with it, consuming O_3 and presumably protecting the underlying cells and the peripheral, more sensitive bronchiolar-alveolar regions of the lung. Peden et al. [23] proposed that uric acid is the

Table I

Oxidation of plasma constituents by ozone

Experimental protocol	Parameters measured								
	Ascorbic acid (μM)			Uric acid (μM)			Protein thiols (μM)		
	$t = 0$	$t = 1$ h	% loss	$t = 0$	$t = 1$ h	% loss	$t = 0$	$t = 1$ h	% loss
(1) Control exposure to O_3	60	9	85	317	92	71	442	381	14
Uricase treated	69	9	87	48	0	100	423	390	8
Supplemented with 250 μM ascorbate	305	44	86	373	88	76	430	367	15
(2) Control Exposure to O_3	84	30	64	445	329	26	–	–	–
Supplemented with 1 mM uric acid	84	47	44	1445	409	72	–	–	–
Supplemented with 1 mM ascorbic acid	1084	440	59	445	233	52	–	–	–
(3) Control exposure to O_3	59	23	61	396	188	53	476	400	16
Supplemented with 1.2 mM ascorbic acid	1214	346	71	409	149	64	–	–	–
Supplemented with 1 mM uric acid	36	14	61	1462	942	36	459	412	10
Supplemented with both 1.2 mM ascorbic and 1 mM uric acids	1258	780	62	1266	675	47	–	–	–

Freshly-prepared human plasma from 3 different subjects was exposed to 16 ppm O_3 for the times stated. Where indicated, ascorbic acid or uric acid were added to give the final concentration stated.

antioxidant present in greatest concentration in human nasal secretions, and is secreted in greater amounts in response to irritants. Hatch [8] has shown that the human upper RTLFs contain little ascorbate or GSH, but considerable uric acid. We therefore propose that one physiological function of uric acid in upper RTLFs is to scavenge inhaled O_3. This may be important because human upper RTLFs contain little ascorbate [8] and the content of albumin (and hence presumably of protein thiols) in RTLFs is much lower than in plasma [15].

It is frequently assumed that lipids are the major target of O_3-induced damage in vivo [1,2,6]. Our data suggest otherwise. Exposure of plasma to 16 ppm O_3, even for 6 h, produced little evidence of lipid damage, measured as aldehydes, lipid hydroperoxides or changes in lipoprotein electrophoretic mobility. Lipid peroxidation might be expected to produce losses of α-tocopherol and bilirubin, which were not observed. Oxidizing agents can damage many other molecules, including proteins and DNA [36–38], and such damage is often more important than damage to lipids during oxidative stress. Indeed, we have recently found evidence for oxidative protein damage in O_3-exposed plasma [39].

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