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Differential Depletion of Human Respiratory Tract Antioxidants in Response to Ozone Challenge

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The toxicity of ozone, the major component of photochemical smog, is related to its powerful oxidising ability, and many of its deleterious effects are mediated through free radical reactions. As the majority of ozone oxidation events are thought to be confined to the pulmonary epithelial lining fluid, we studied the interaction of ozone with a range of small molecular weight antioxidants found within this compartment: ascorbic acid (AH_2), uric acid (UA), and reduced glutathione (GSH). Epithelial lining fluid obtained as bronchoalveolar lavage (BAL) fluid, was taken from 16 male subjects and the antioxidant concentrations determined for each subject. BAL fluid samples from nine of these subjects were then exposed, using an interfacial exposure system, to a range (50–1000 ppb) of ozone concentrations. Both AH_2 and UA were consumed by ozone in a time and ozone concentration dependent manner, with mean consumption rates of 1.7 ± 0.8 and 1.0 ± 0.5 pmol L^{-1} s^{-1} ppb^{-1}, respectively. Considerable intersubject variation was however observed. The individual rates of consumption for each antioxidant were significantly correlated with the respective initial antioxidant concentration. In contrast, although GSH was consumed at 50 ppb ozone, the rate of consumption did not change with increasing ozone concentration. We conclude that there is differential depletion of BAL fluid antioxidants, suggesting a reactivity hierarchy toward ozone in human ELF of AH_2 > UA ≫ GSH.

Keywords: Ozone, antioxidant, epithelial lining fluid, lung, uric acid, ascorbic acid, glutathione

INTRODUCTION

Inhalation of ozone (O_3), at concentrations two or three fold greater than normal environmental background levels, has been demonstrated to elicit a broad spectrum of pathophysiological effects in human subjects.[1–4] Despite the extensive literature describing these effects, relatively little is appreciated about the underlying mechanisms; especially how the initial oxidative insult is manifest as acute and chronic lung injury. The external surfaces of the respiratory tract including the nasal passages, trachea, conducting air-

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ways and alveoli are covered with a thin layer of aqueous material, the epithelial lining fluid (ELF).[5–6] This aqueous hydrophase represents the first physical interface encountered by inspired ozone. ELF is invested with a range of antioxidant compounds, including the small molecular weight antioxidants, uric acid (UA),[7] ascorbic acid (AH_2)[8] and reduced glutathione (GSH).[9] These compounds are present in high concentrations and display high intrinsic reactivities toward ozone.[10–15]

It has been predicted from mathematical models of the transit time of O_3 through the ELF, based upon its reactivity toward the substrates within this compartment,[10] and its rate of diffusion through aqueous media,[11] that only a small fraction (10%) of the O_3 incident at the surface of the ELF will penetrate this layer to react with the underlying epithelium.[12] Consequently, the toxic effects of O_3 are thought to be predominately mediated through the action of secondary and tertiary ozonation products in a mechanism referred to as the *cascade mechanism of toxicity*.[16] This hypothesis has gained support from the observations of Postlethwaites' group[17,18] who have reported that the absorption of O_3 into this aqueous compartment is mediated through reactions with antioxidants in the ELF at the gas/liquid interface. This mechanism, coined *reactive absorption*, was derived from studies in which the fractional uptake of O_3 into pure biochemical antioxidant solutions or rat BAL fluid was measured.

Clearly, to understand O_3 toxicity in humans, knowledge of the components of this aqueous compartment and the nature of their interaction with O_3 is critical to an understanding of O_3 toxicity. In the present study, we measured the consumption of antioxidants in human ELF by O_3 in order to delineate the relative importance of these species as substrates for O_3. Using bronchoalveolar lavage (BAL) fluid as a diluted model of ELF we found that contrary to what would be expected from the published bimolecular rate constants for GSH,[19] UA[13] and AH_2[13] that GSH did not represent a significant substrate for ozone, and that AH_2 was the most important scavenger of O_3 in lung ELF. This apparent lack of reactivity of GSH towards O_3 was in agreement with other studies[15,17] in which the reactivity of GSH has been shown to be significantly smaller in interfacial exposure models compared with the rates observed in bulk phase mixing models.[19]

MATERIALS AND METHODS

Subjects and Bronchoalveolar Lavage (BAL) Procedure

BAL fluid was collected by fibreoptic bronchoscopy from 16 male patients under investigation for suspected lung carcinoma. Samples were selected on the basis of sufficient recovery and the absence of blood contamination. Bronchoscopy was performed as described previously.[20] Briefly, after topical anaesthesia of the upper respiratory tract with 2% lidocaine, an Olympus BF Type 20 bronchoscope was wedged in the right middle or left lingula lobe of the lung at the third or fourth airway generation. Warmed (37°C), sterile saline was introduced into the lower respiratory tract, in four sequential 50ml aliquots, which were gently aspirated individually into a sterile trap, pooled and kept briefly at 4°C. The lavage was filtered through coarse (0.74 mm) plastic mesh to remove mucus. The filtered lavage fluid was centrifuged at 300 g for 10 min at 4°C to separate the BAL cells from the acellular BAL fluid. The BAL supernatants were stored in 10 ml aliquots at –70°C for up to 6 weeks prior to antioxidant measurement and subsequent ozone challenge. Antioxidants, by their very nature are prone to autoxidation reactions especially AH_2 in the presence of transition metals.[21] In order to minimise the likelihood of such interactions occurring BAL fluid was treated with deferoxamine mesylate and EDTA to give a final concentration of 0.1 mmol/L. This approach has been adopted in a number of other studies.[22,23] The pH of BALF samples was also

adjusted to 7.4, which is reflective of the pH of normal airway secretions *in vivo*.[24]

Exposure Protocol

Sufficient BAL fluid was available from 9 subjects for the ozone exposure protocol. Exposure of BAL fluid was carried out in a 5.6 litre purpose-built Perspex chamber, flushed with ozone at 50, 100, 250 or 1000 ppb at a flow rate of 3 litres/min. The main features of this exposure system have been described previously.[23] We employed inter-facial exposure as this more closely approximates the *in vivo* situation than a bubble through system and because considerable differences between ozone/antioxidant interactions have been reported between the 2 modes of exposure.[15] In addition, BAL fluid was mixed throughout the exposure period. This was done to compensate for the difference in the available reactive surface area between our system and the lung. Mixing meant that substrate limitation at the BAL fluid/ozone interface was unlikely to become a limiting factor, and was preferred to increasing the ambient ozone concentration, as it was believed that interfacial resistance could result in anomalous reactions occurring at the gas/liquid interface. Temperature was maintained at 37°C throughout the exposure. At 30, 60, 120 and 240 minute intervals throughout the exposure, the chamber was opened briefly, samples were removed and snap frozen in liquid nitrogen prior to storage at –70°C. Evaporative loss from the samples during the four hour exposure was minimal. However, corrections for any concentrating effect were achieved by using a correction index derived from the apparent increase in glutathione disulphide (GSSG) concentration in a series of GSSG samples (200μmol/L in 0.9% saline, pH 7.4). These were run in parallel to, and exposed in an identical manner to the BAL fluid samples. GSSG has been demonstrated not to act as a substrate for O_3 in gaseous absorption models.[17]

Antioxidant Determinations

GSH, GSSG, UA and AH_2 were determined as described previously.[23] Briefly, UA and AH_2 were measured simultaneously using reverse phase chromatography, using a 25 cm Apex II ODS column and a mobile phase of 0.2 mol L^{-1} KH_2PO_4, 0.25 mmol L^{-1} octane sulphonic acid (pH 2.1). Electrochemical detection was employed (using a current sensitivity of 0.1 nAmps) and concentration was determined with reference to a standard curve of 0 – 6.3 and 0 – 12.5 mmol L^{-1}, for AH_2 and UA respectively. The assay sensitivities for AH_2 and UA were 0.01 and 0.005 mmol L^{-1}, respectively. GSH and GSSG were determined using the enzyme recycling assay modified for use on a plate reader. Final concentrations were determined with reference to standard curves of 0–330 μmol L^{-1} and 0–165 μmol L^{-1} GSH and GSSG, respectively. Assay sensitivities were 0.05 and 0.01mmol L^{-1}, GSH and GSSG respectively. All antioxidant analyses were carried out within 24 hours of exposure to ozone.

Statistical Treatments

Group distributions were determined using the Kolmogorov Smirnov test of normality. This revealed that the BALF antioxidant concentration data was predominately non-parametric, and therefore the concentrations are expressed as median values with interquartile range. Antioxidant consumption data, expressed either in percentage terms, or as a rate, were found to be representative of samples drawn from normally distributed populations. Statistical analysis of these data sets was therefore conducted using a two-way analysis of variance with the Bonferroni correction for repeated measures. Duration of exposure (0, 30, 60, 120, 240 minutes) and ozone concentration (50, 100, 250 and 1000ppb) were factors for analysis of the percentage change in 'antioxidant concentration' data set, whilst antioxidant (UA, AH_2, GSH) and ozone concentra-

tion (as above) were factors for analysis of the 'antioxidant consumption rate' data set. The Student-Newman-Keuls procedure was used to compare all paired group means. Where comparison between two isolated group means was necessary, this was achieved using Students unpaired *t* test. Bivariate correlations were performed using the Pearson correlation coefficient, where both variables were drawn from normally distributed populations. Where one, or both of the variables failed to satisfy the assumption of normality, Spearmans rank order correlation was utilised. In cases where individual bioariate correlations indicated a potential spurious interaction between variables, the true predictor was determined using partial correlation analysis. Linearity of response was determined by linear regression analysis using the least square method. Statistical significance for all comparisons of means, correlations and linear regression analysis was accepted at $P < 0.05$.

RESULTS

Bronchoalveolar Lavage Fluid Antioxidant Status

Baseline antioxidant and protein concentrations were determined in BALF samples obtained from the 16 subjects recruited to the study (Table 1). UA was detected in BALF from all subjects at a median (interquartile range) concentration of 1.4 (0.8–3.3) μmol/L; displaying a 32-fold difference between the minimum and maximum recorded values. GSH was present in all BALF samples at a concentration of 2.9 (1.9–4.1) μmol/L. GSSG was present in the majority (14/16) of individuals and it represented a significant fraction of the total glutathione pool at 22.2 (11.1–36.5)%. AH_2 was present in only 5/16 BALF samples examined: 0.01 (0.01–1.77) μmol/L. BALF protein concentrations varied little 0.41 (0.26–0.55) mg/ml between subjects.

TABLE I Individual BAL fluid antioxidant concentrations

Subject No	Protein (mg/mL)	GSH (μmol/L)	GSSG (μmol/L)	AH_2 (μmol/L)	UA (μmol/L)
1	0.2	1.5	0.2	nd	2.0
2	0.6	1.9	0.3	nd	3.8
3	0.5	4.6	1.5	nd	7.4
4	0.5	6.2	2.9	2.7	6.1
5	0.2	2.0	0.1	nd	3.5
6	0.6	3.4	nd	1.4	1.1
7	0.6	2.8	0.7	2.1	3.1
8	0.6	2.4	nd	3.4	0.7
9	0.6	2.9	0.1	4.4	0.8
10	0.3	3.6	2.1	nd	1.9
11	0.2	2.6	1.4	nd	1.7
12	0.4	1.9	0.4	nd	0.8
13	0.3	3.9	1.6	nd	1.1
14	0.2	4.2	0.4	nd	0.2
15	0.3	1.2	0.3	nd	0.7
16	0.5	6.4	1.4	nd	1.1
median	0.5	2.9	0.4	0.01	1.4
25 percentile	0.3	2.0	0.2	0.01	0.8
75 percentile	0.6	4.1	1.3	1.2	3.3

Note: Values are not corrected for BAL recovery.
nd: not detected.
Samples 1–9 were utilised for ozone exposure studies.
Where no sample was detected the lowest detectable concentration using each assay was inserted for the generation of the group median values: 0.01 μmol/L for both GSSG and AH_2.

Ozone-Induced Changes in BALF Antioxidant Concentrations

UA consumption was not observed when BALF was exposed to 50 ppb ozone (Figure 1a). Loss of UA was detected under the 100 ppb ozone exposure protocol; the concentration falling to 68 ± 18 and 35 ± 25% of the initial concentration, by 120 and 240 min, respectively. At 1000 ppb ozone, all UA was depleted within 120 min of exposure (Figure 1a). In contrast to UA, some AH_2 depletion was seen at 50 ppb ozone, 83.7 ± 2.9% remaining after 60 min of exposure (Figure 1b). Overall, the rate of AH_2 consumption by ozone was more rapid than that of UA under equivalent exposure conditions. For example, during exposure to 1000 ppb ozone. AH_2 was completely consumed after 30 min exposure. Both AH_2 and UA antioxidants were consumed in a time and ozone concentration dependent manner. In contrast, GSH only showed a time-dependent decrease in concentration. The rate of GSH consumption did not change as the ozone concentration increased such that the percentage GSH in the BAL fluid samples remained at approximately 50% of baseline under 50, 100, 250, and 1000 ppb ozone, after 240 min of exposure: 62.7 ± 14.3; 56.8 ± 12.5; 50.8 ± 26.0; and 67.2 ± 16.6% respectively (Figure 1b). No change in GSSG concentrations was detected under any of the exposure conditions employed (Figure 1b).

Antioxidant Consumption Rates

To clarify the relationship between the rate of UA, AH_2 and GSH consumption, individual rates of change in antioxidant concentration with time were determined for each antioxidant. Data for subject '4' are presented in Figure 2, as these results are indicative of those obtained for all the other BAL fluid samples. A linear pattern of antioxidant consumption with time is clearly evident. The strength of this relationship indicates that the reaction is zero order with respect to the antioxidant concentration, i.e., the rate at which the antioxidant is consumed is independent of the antioxidant concentration itself.

Consumption rates were determined from the gradients of these linear regressions and were expressed as nmol L^{-1} s^{-1}. Where the data could not be described by the linear model, data points were described using a polynomial function, and the rate determined over the linear portion of the graph. Under the most extreme exposure conditions, where antioxidant concentrations were completely consumed within the first 30 min, an approximation of the consumption rate was obtained by assuming a linear relationship between the initial concentration at time 0, and a concentration of zero at 30 min. This compromised approach generated a conservative estimate of the rate, which is overly sensitive to the initial ozone concentration. The mean consumption rate data for each antioxidant for all subjects is summarised in Table 2. AH_2 was consumed at a significantly greater rate than GSH at 250 ppb ozone and GSH and UA at 1000 ppb ozone. The consumption of UA was also significantly greater than GSH at 1000 ppb suggesting at 1000 ppb, an overall ozone reactivity hierarchy in which AH_2 > UA ≫ GSH.

To further define the relationship between the rate of antioxidant consumption in BAL fluid and ozone concentration, a plot of the individual rates of AH_2, UA and GSH consumption against ozone concentration was performed. These results demonstrated a highly linear positive relationship in each individual BAL fluid sample with respect to both AH_2 and UA (Figure 3). No such relationship was apparent for GSH. The reactivity of each antioxidant toward ozone was determined from the gradient of the regression line for each individual BAL sample (Table 3). Of the 9 BAL samples in which UA consumption was followed, 7 displayed a highly significant linear association between consumption rate and ozone concentration. In the remaining 2 samples (subjects 1 and 5), the data did not fit the linear model, and an overall rate was determined from the linear portion of a polynomial regression line.

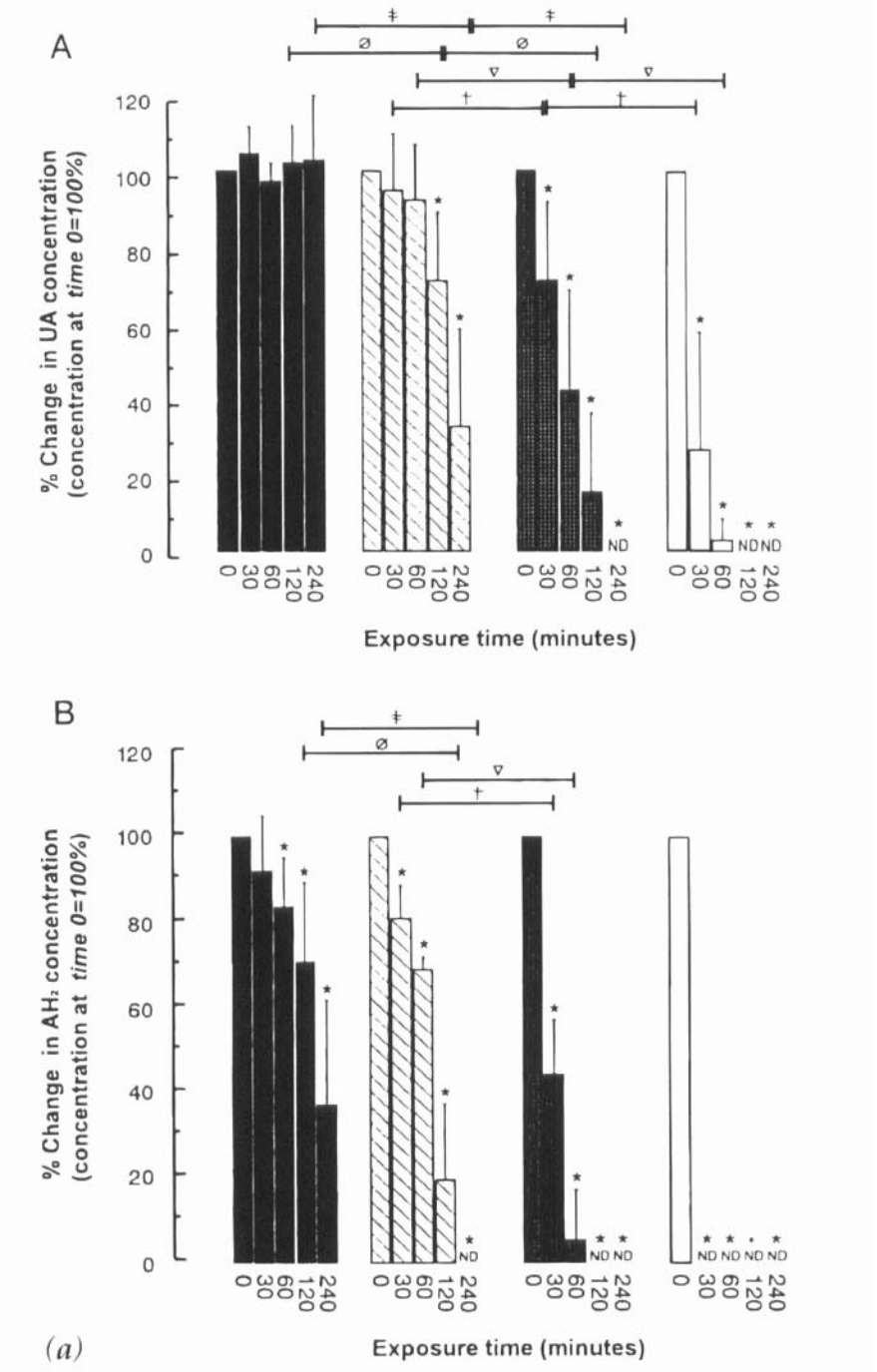

FIGURE 1a Consumption of BAL fluid uric acid and ascorbic acid (panels 'A' and 'B' respectively) under a range of ambient ozone concentrations: 50 ■ ; 100 ■ ; 250 ■ and 1000 □ ppb. Results are expressed as the percentage change in antioxidant concentration, assuming the concentration at the time 0 (prior to ozone exposure) to be 100%, and are given at 30, 60, 120, and 240 minutes into the exposure period. Results are expressed as the mean ± standard deviation, n=9 with respect to the uric acid data and 5 for ascorbic acid.'*' indicates a significant difference in the mean values between time points at a given ozone concentration. Significant differences between ozone treatments at equivalent time points are indicated as: † for 30; ▽ for 60; Ø for 120; and ≠ for 240 minutes. Significance was assigned at the $P < 0.05$ level.

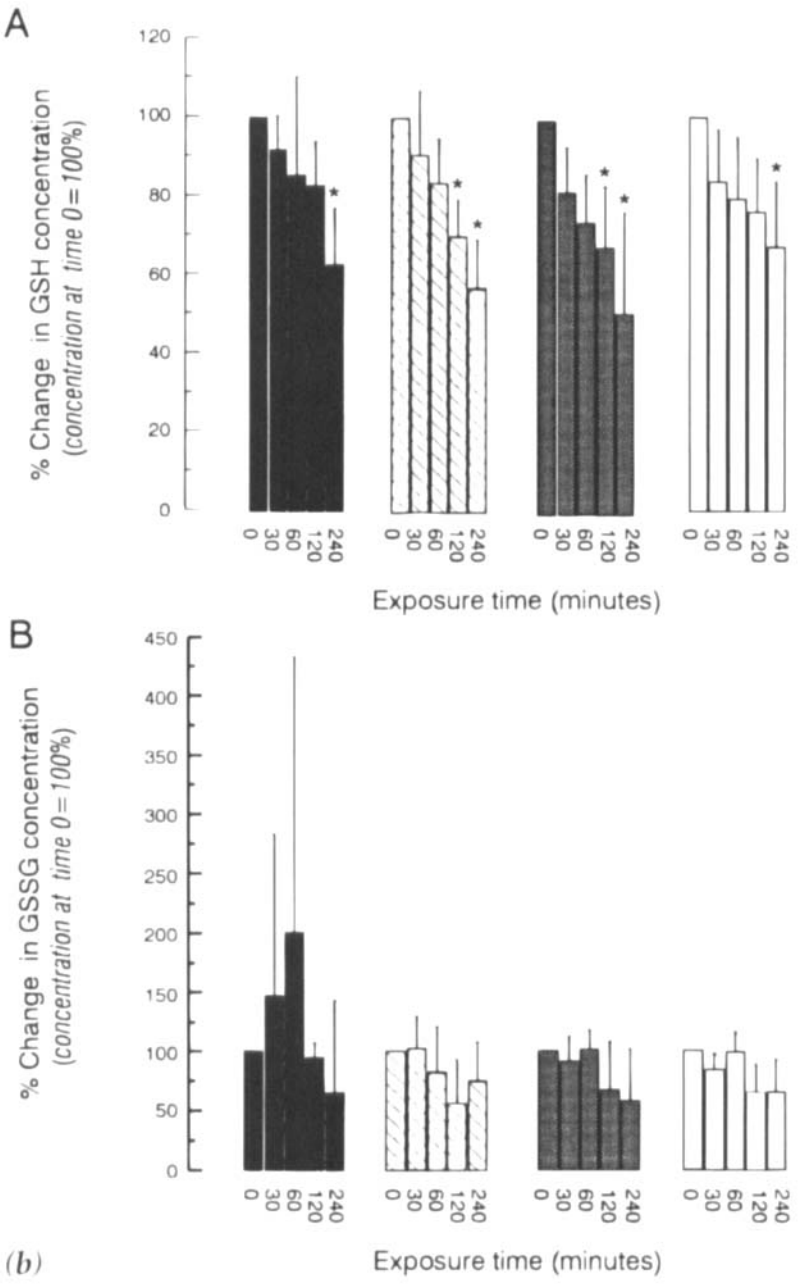

FIGURE 1b Changes in bronchioveolar lavage fluid reduced and oxidised glutathione concentrations (panels 'A' and 'B' respectively) under a range of ambient ozone concentrations: 50 ■, 100 ▧, 250 ■ and 1000 □ ppb. Results are expressed as the percentage change in concentration, assuming the concentration at time 0 (prior to ozone exposure) to be 100%, and are given at 30, 60, 120, and 240 minutes into the exposure period. Results are expressed as mean ± standard deviation. n=5. '*' indicates a significant difference in the mean values between time points at a given ozone concentration.

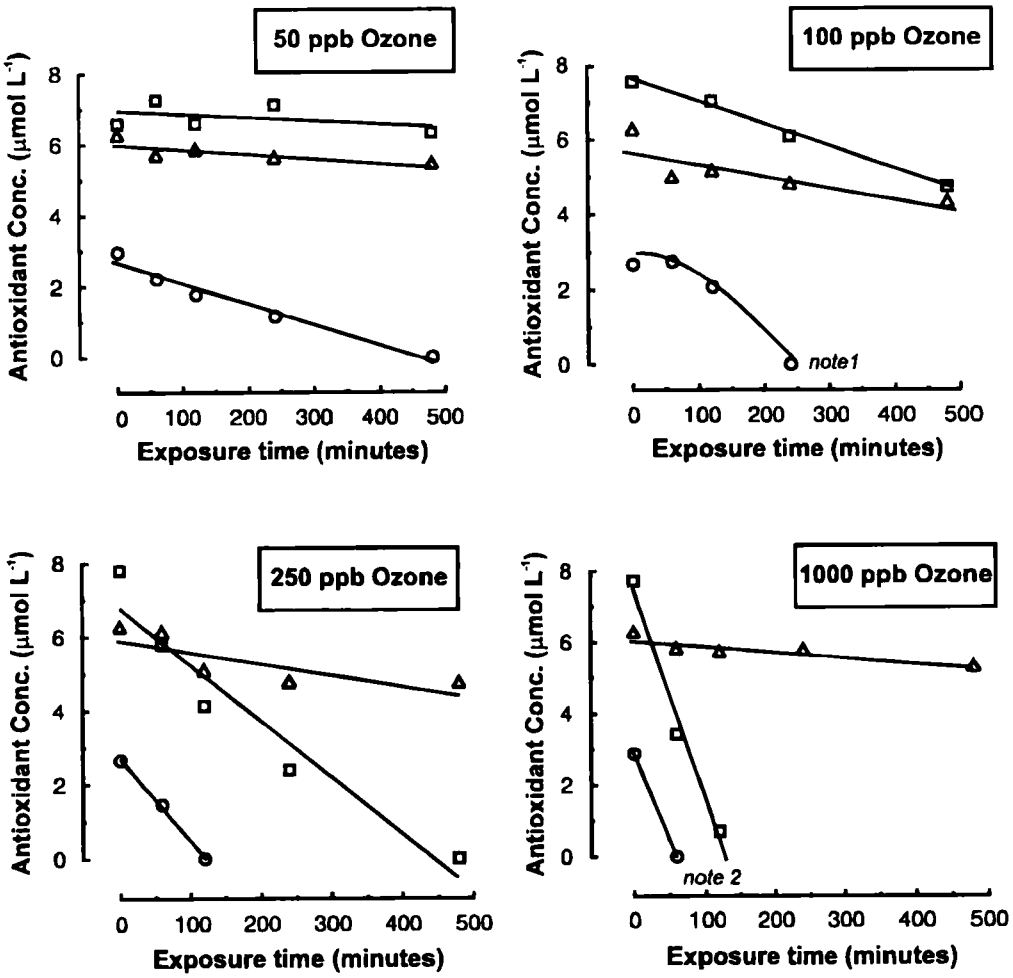

FIGURE 2 Consumption of BAL fluid antioxidants: open squares , uric acid; open triangles, reduced glutathione; and open circles, ascorbic acid from subject 4, under a range of ambient ozone concentrations. Each point represents the mean of three values. Details of the regression lines used for the determination of consumption 'rates' (reaction velocities) were as follows:

O_3 conc. (ppb)	Uric acid		Ascorbic acid	Reduced glutathlone	
50	r: – 0.929	p: 0.050	r: – 0.996 p:0.045	r: – 0.821	p: 0.088
100	r: – 0.985	p: 0.003	*c. note 1*	r: – 0.972	p: 0.041
250	r: – 0.964	p: 0.008	r: – 0.999 p: 0.034	r: – 0.769	p: 0.129
1000	r: – 0.992	p: 0.052	*c. note 2*	r: – 0.884	p: 0.046

The final rates for all subjects are summarized in table '3'.
Both note '1' and note '2' refer to conditions under which the data did not fit the linear model and where alternative treatments were employed to derive a final consumption rate.
These treatments are described in detail in the results section.

TABLE II Antioxidant consumption rates in human BAL fluid exposed to a range of ambient ozone concentrations.

		nmol L–1 sec–1			
Antioxidant	n	50 ppb O_3	100 ppb O_3	250 ppb O_3	1000 ppb O_3
AH_2	5	0.1 ± 0.1	0.3 ± 0.1	0.7 ± 0.3*‡	1.8 ± 0.8*†‡
UA	9	0.01 ± 0.02	0.2 ± 0.2	0.4 ± 0.2	0.9 ± 0.5*¶
GSH	5	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.03	0.1 ± 0.03*¶

Note. Values are means ± standard deviation
'*' indicates a significant difference between the consumption rate of a particular antioxidant under different ozone concentrations; '†' a significant difference between the rate of ascorbate and urate consumption at a given ozone concentration; '‡' between ascorbate and reduced glutathione; and '¶' between urate and reduced glutathione. Significance is assumed in all cases when $P < 0.05$.

The overall rate of UA consumption by ozone was determined as 1.0 ± 0.5 pmol L^{-1} s^{-1} ppb^{-1}, and displayed considerable intersubject variation; with a 5.2 fold difference between the maximum and minimum calculated rates. When the overall consumption rate of the UA in the BALF samples containing AH_2 (1.0 ± 0.4 pmol L^{-1} s^{-1} ppb^{-1}; n = 4) was compared against the rate observed in the subset lacking AH_2, (0.9 ± 0.7 pmol L^{-1} s^{-1} ppb^{-1}; n = 5) no significant difference could be detected. The relationship with respect to the consumption of AH_2, under a range of ozone concentrations, was found to conform to the linear model. A consumption rate of 1.7 ± 0.8 pmol L^{-1} s^{-1} ppb^{-1} was calculated for AH_2, with a 3.4 fold difference between the minimum and maximum rates. Due to the variation between individual AH_2 and UA consumption rates, no significant difference between the two was evident. (P = 0.06).

Correlation Analysis

The degree of association between UA and AH_2 consumption, and individual BAL fluid antioxidant status, was assessed in order to dissect out any underlying basis for the inter-individual variation in antioxidant consumption rates. Both the individual rates of AH_2 and UA consumption were found to be positively associated with their initial concentrations in BAL fluid, r: 1.0, *P*: <0.001; and, r: 0.85, $P < 0.005$, respectively (Figure 4). The rate of UA consumption was also found to be correlated to the initial GSSG and GSH concentrations: r: 0.85. P: <0.0001; and, r: 0.81, P: <0.01, respectively. However, when these correlation's were corrected for the underlying association between baseline GSH and GSSG concentrations, only the interaction with initial GSSG concentration remained significant: r: 0.85, P: 0.008 (Partial correlation analysis; Figure 5). No interaction was apparent between the rates of AH_2 and UA consumption, suggesting that their consumption was independent of each another. No such correlation was seen between AH_2 and GSH or GSSG.

DISCUSSION

The primary aim of the study was to model, as closely as possible, the nature of the interactions occurring between ozone and the antioxidant constituents of the pulmonary ELF characteristic of the distal portions of the lung. The information obtained will help elucidate the relative importance of these biomolecules in conferring protection against O_3-induced tissue injury. Events occurring within the distal portions of the tracheo-bronchial tree were considered important, as this region is believed to receive the greatest dose of inhaled ozone.[25–26] Furthermore, ozone has been shown to produce site specific lesions and epithelial remodelling predominantly within these regions.[27–29]

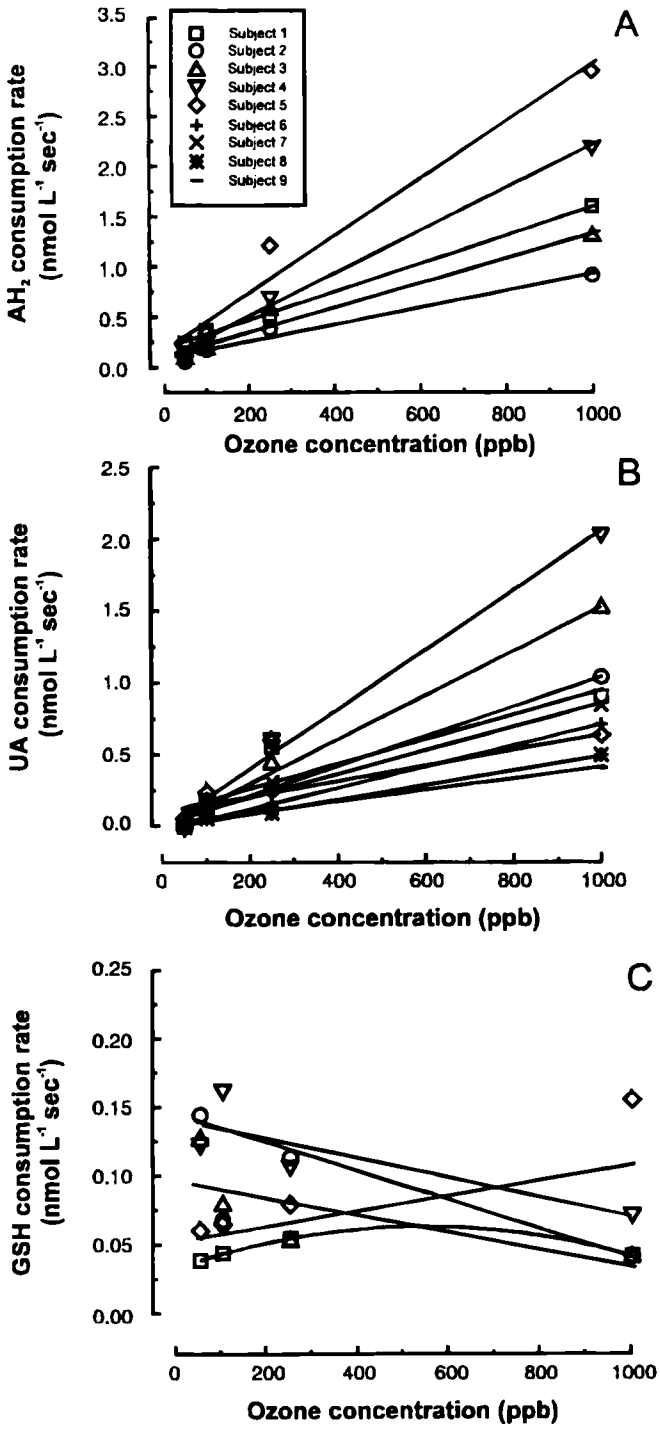

FIGURE 3 Relationship between antioxidant consumption rates in individual BALF samples and ambient ozone concentration with respect to: 'A' ascorbic acid; 'B' uric acid and 'C' reduced glutathione.

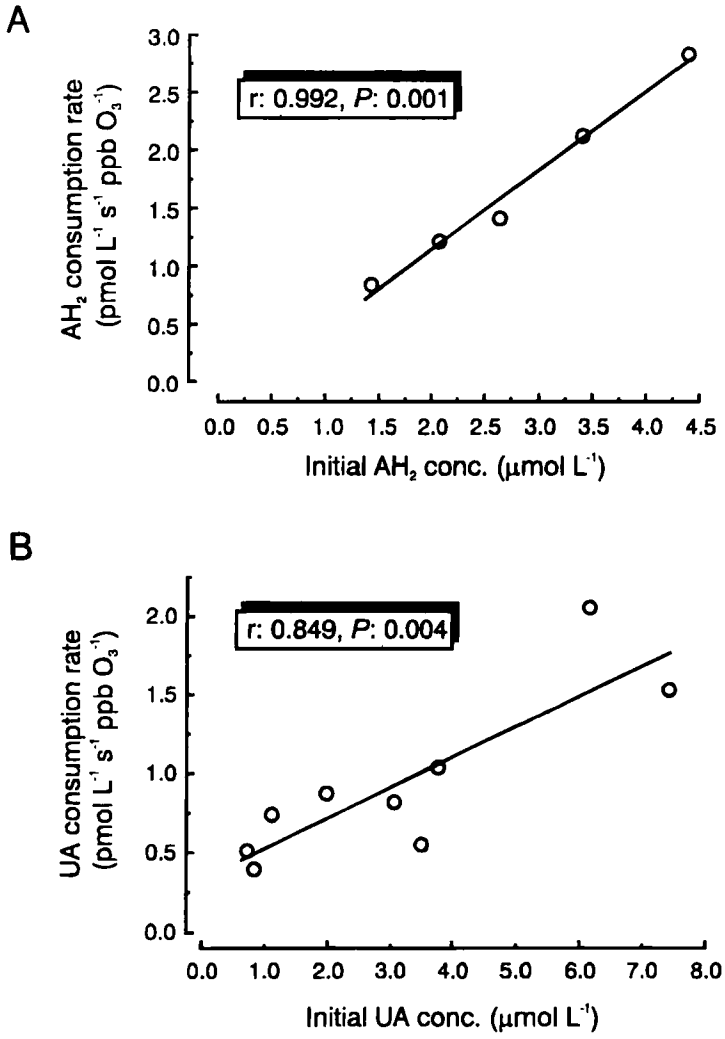

FIGURE 4 Correlations between the overall UA and AH_2 individual consumption rates with their respective initial BALF concentrations.

The ozone concentrations we employed include 50 ppb to represent a high ambient background (environmental baseline ozone concentrations typically vary between 25–35 ppb[30]), 100 ppb as a typical peak concentration, 250 ppb as an extraordinary but attainable environmental concentration and 1000 ppb to help paramatise the kinetic data. BAL fluid was utilised as a diluted surrogate for pulmonary ELF, as it represented the best available model of the lung lining fluids in the relevant portion of the respiratory tract, being predominately representative of the distal lung lining fluid.[31] Furthermore, Postlethwait has demonstrated that the reaction of ozone and nitrogen dioxide with airway substrates (measured as the fractional uptake of ozone or nitrogen dioxide) is comparable between rat BAL fluid and isolated perfused and un-perfused rat lungs, indicating important similarities between lung lining fluid and BAL fluid.[17,18,32,33]

Where comparison between individuals is necessary, the use of BAL fluid further compli-

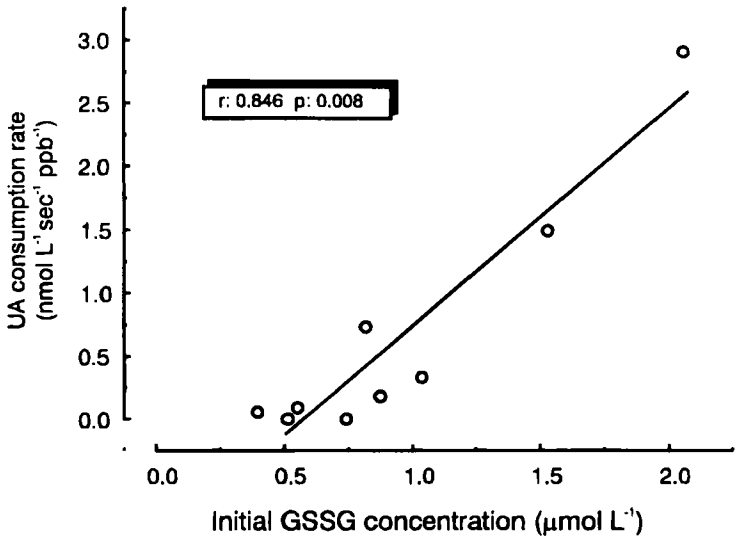

FIGURE 5 Correlation between the overall UA individual consumption rates and the initial GSSG concentration.

cates data interpretation as considerable variation in dilution may occur between individual procedures, coupled to the fact that there is no universally acceptable method for correcting for this variation.[5,6] In the present study, this does not represent a significant limitation, as our aim was to study the relative change in antioxidant concentration from a known baseline and it was therefore not necessary to relate this back to the situation in lung ELF. It should be appreciated that the BAL fluid samples employed may have contained trace amounts of Lidocaine which has some antioxidant properties. We believe however, that the contamination to be minimal and probably consistent between samples.

UA and GSH were detected in BAL fluid from all 16 subjects in the present study. In contrast AH_2 was only detected in 5/16 subjects, which was at variance, both with the findings of Hatch[34] and with our own previous observations, in which AH_2 has been ubiquitously detected in over 200 BAL fluid samples.[35] This discrepancy may be explainable, both in terms of the age range of the subjects examined (32–84 years; compared with 18–35 years in the Hatch study, and 18–31 years in our own work) and the fact that most were current smokers, or had a recent history of smoking. Serum AH_2 concentrations are depressed in smokers, while GSH concentration are elevated in smokers ELF,[9] both of which are consistent with our observations. The high concentrations of recoverable protein and GSSG are also characteristic of a lung which has been placed under oxidative stress. The fact that these BAL fluid samples could not therefore be regarded as 'normal' was not an impediment to the second part of this study. Sufficient antioxidant was present in its reduced form to allow their oxidation by ozone to be followed. Furthermore, the presence of AH_2 in only 5 samples subsequently exposed to ozone, allowed the rate of UA to be compared in both the presence and absence of AH_2 without having to artificially modify the BALF sample.

The data obtained support the reaction scheme $AH_2 > UA \gg GSH$ when BAL fluid is exposed to ozone. This differs markedly from the scheme predicted from the rate constants of these water soluble antioxidants reactions with ozone. For example, although UA has been

shown to have a well defined antioxidant function *in vitro*,[36,37] its bimolecular rate constant with respect to ozone at pH 7.0, 1.4×10^6 $M^{-1}s^{-1}$ is considerably lower than that for AH_2, 6×10^7 $M^{-1}s^{-1}$,[13] and GSH, 7×10^8 M^{-1} s^{-1},[19] and not much greater than that of unsaturated fatty acids.[12] These rate constants, determined using a combination of stop-flow kinetic measurements under acidic conditions, predict a reaction hierarchy of GSH >> AH_2 >> UA toward ozone. In contrast, we found little evidence that GSH acted as a good substrate for ozone. Similarly, and supporting our findings, the rate of protein sulfhydryl consumption by ozone was a fraction of that of AH_2 and UA in ozone-exposed plasma.[38]

The lack of ozone reactivity toward thiol groups is puzzling given its purported reactivity. GSH oxidation products can be detected in erythrocytes exposed to ozone,[39] and addition of cysteine to solutions of tryptophan prevents its oxidation, suggesting that it can act as a preferred substrate for ozone.[40] Furthermore, GSH has been shown to be a major substrate for nitrogen dioxide, another gaseous pollutant causing concern at present.[33] By way of explanation, the bimolecular rate constant for GSH, and thiols in general, may have been over estimated.[15,41] Using the rate of ozone absorption into solutions containing defined substrates, Kanofsky and colleagues[41] determined the bimolecular rate constant to be 2.5×10^6 $M^{-1}s^{-1}$ for GSH. This rate is more consistent, with our findings, being an order of magnitude lower than AH_2. It is still however larger than that of UA and can not therefore explain why UA is consumed, whilst GSH is not, given that the two are present in roughly equimolar concentrations. Further evidence supporting the lack of reactivity of GSH in BAL fluid has been reported by Langford and colleagues.[17] Using the rate of fractional uptake of ozone, or reactive absorption, into rat BAL fluid in a mixed interfacial exposure system, they demonstrated that removing GSH by NEM only marginally (by 2–10%) reduced the overall fractional uptake of ozone. In addition, with pure biochemical solutions of AH_2, UA and GSH, we have shown that while AH_2 and UA are consumed at an equivalent rate, the consumption of GSH is significantly slower.[42]

Postletwaite and colleagues[33] reported AH_2 to be an important ozone reactive substrate in rat BAL fluid, concurring with our observation of its significance in human BAL fluid. They reported that treatment with ascorbate oxidase abolished approximately 30% of the fractional ozone uptake observed. In addition, they found that when they dialysed rat BAL fluid against a 1000 KDa cut off filter, the small organic moieties only contributed 30% of the overall absorption. From these data they concluded that ozone reacts predominately with macromolecular components present in BAL fluid. In light of our results, we believe that they may have underestimated the significance of the small organic moieties. This may have arisen because all the AH_2 was consumed by 1000 ppb ozone during the initial (30 min) sampling point. In such a case, the rapid loss of AH_2 from BAL fluid will lead to an underestimation of its significance as a reactive substrate.

In summary, this study has demonstrated that when BAL fluid is exposed to environmentally relevant concentrations of ozone, there is a differential consumption of water soluble antioxidants in the order AH_2 > UA >> GSH. These rates of consumption are consistent with their functioning as a protective sacrificial substrates. Although these are not the only antioxidants present within the lung epithelial lining fluid compartment, their high reactivity towards ozone indicates that they form an important first line of defence against ozone.

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