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OZONE-INDUCED TISSUE INJURY AND CHANGES IN ANTIOXIDANT HOMEOSTASIS IN NORMAL AND ASCORBATE-DEFICIENT GUINEA PIGS*

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Abstract—It has been reported previously that ozone (O_3) toxicity from acute (4 hr) exposure is enhanced by ascorbate (AH_2) deficiency in guinea pigs. We hypothesized that lung injury from continuous 1-week O_3 exposure would also be increased under conditions of AH_2 deficiency because of (1) a diminished antioxidant pool to counteract the oxidant challenge, (2) impaired reparation of tissue injury, and/or (3) altered antioxidant redox homeostasis. Female Hartley guinea pigs (260–330 g) were made AH_2 deficient by providing a diet similar to guinea pig chow, but having no AH_2. The dietary regimen was started 1 week prior to exposure and was continued during exposure to O_3 (0, 0.2, 0.4, or 0.8 ppm, 23 hr/day, 7 days) as well as 1 week post-exposure. Bronchoalveolar lavage (BAL) and tissue AH_2 were measured in subgroups at the beginning of exposure (1 week on the AH_2-deficient diet), at its termination and 1 week post-exposure. AH_2 measured in ear tissue punches proved to be an easy and effective monitor for AH_2 deficiency. One week on the AH_2-deficient diet caused a 70–80% drop in ear, lung and liver AH_2, while AH_2 in BAL was decreased by 90%. Immediately after the exposure, total BAL protein and albumin (markers of lung permeability) were increased (~50%) at 0.8 ppm with no difference between the dietary groups. O_3 caused an increase in total BAL cells and neutrophils in a concentration-dependent manner with only a slight augmentation due to diet. Exposure to O_3 caused an increase in lung and BAL AH_2 in normal guinea pigs. Glutathione and uric acid were also increased in the lung and BAL after O_3 exposure (40–570%) in both dietary groups, and the levels remained elevated during the recovery period. Lung α-tocopherol was not changed due to O_3. A significant overall diet-related decrease was seen in AH_2-deficient guinea pigs, immediately after the exposure and recovery. In summary, lung injury/inflammation following 1 week O_3 exposure and recovery were minimally affected by AH_2 deficiency. Antioxidants also appeared to increase in response to O_3 exposure despite the deficiency in AH_2.

Key words: ozone; lung; ascorbate deficiency; antioxidants; inflammation; guinea pig

O_3‖, a major oxidant air pollutant, is associated with acute lung functional impairment in humans and is thought to be linked to chronic lung diseases [1, 2]. The O_3-induced lung tissue damage is characterized by neutrophilic inflammation and accumulation of protein in air space lumen [3–6]. Depending upon the concentration and the duration of exposure, adaptive and repair processes are initiated that may be critical to the recovery of lung structural integrity [7, 8]. Several changes associated with adaptation and repair in the lung include: stimulated collagen synthesis [9, 10], induction of several antioxidant enzymes [11, 12], and constitutive increases in antioxidant substances [12, 13].

Antioxidant substances help maintain a critical redox balance in the cell during oxidative challenge. One such antioxidant, AH_2 is present in lung tissue as well as in BAL sampling of lung epithelial lining fluid [14]. It is known to react directly with O_3 *in vitro* [15], while high dose *in vivo* supplementation of AH_2 in the diet has been shown to decrease O_3-induced bronchial hyperreactivity and pulmonary edema in animals [16, 17]. GSH is another antioxidant known to protect animals from O_3-induced lung injury [12, 18]. Because both of these antioxidants are elevated after O_3 exposure, they have been proposed to be a part of an adaptive response [8, 18]. On the other hand, while deficiency in α-tocopherol has been shown to enhance O_3 toxicity in animals [19, 20], its role in adaptation remains unclear.

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‖ Abbreviations: O_3, ozone; BAL, bronchoalveolar lavage; AH_2, ascorbate, vitamin C; GSH, glutathione; IgG, immunoglobulin G; OPD; *O*-phenylenediamine dichloride; and MANOVA, multivariate analysis of variance.

Similarly, uric acid, which is also reactive with O_3 [21], has been shown recently to be present in high concentrations in the upper and lower airways, contributing to the antioxidant status of lung lining fluid [22]. Antioxidants, therefore, appear to play a major role in defense against toxicant challenge and may contribute to adaptation response.

In the U.S., individuals residing in urban areas are likely to endure relatively high O_3 exposure. Nutritional antioxidant deficiency may alter the sensitivity of individuals to O_3-induced lung injury [23]. Although dietary antioxidant supplementation is increasingly popular, antioxidant deficiency associated with poor nutrition in humans may still pose a significant health problem [24–26]. Among the antioxidant substances, AH_2 is an essential nutrient of the human diet necessary for tissue repair and has been associated with adaptation to continued O_3 exposures [8]. In a rat model, adaptation in lung function measurements after repeated intermittent O_3 exposure without apparent adaptation in BAL protein and histopathology has been correlated with increases in lung AH_2 [8]. Consistent with the protective role of AH_2, we have reported that injury induced by acute (4 hr) O_3 exposure (as measured by an increase in BAL protein) is enhanced markedly by AH_2 deficiency in a guinea pig model [27], which, like humans, lacks the ability to synthesize AH_2. While attenuation in lung function to intermittent O_3 exposure in a rat model is correlated with increases in AH_2 [8], our related study of a 1-week continuous O_3 exposure regimen in guinea pigs indicated that AH_2 deficiency causes only a minimal exaggeration of O_3-induced abnormalities in lung functions [28].

In this study we examined the impact of AH_2 deficiency on the lung inflammatory response after 1 week of continuous O_3 exposure when antioxidant induction and stimulated repair in response to O_3 would be anticipated. We hypothesized that since AH_2 is both an important airway antioxidant and an essential component of tissue repair, its deficiency would exacerbate O_3 effects observed after 1 week of exposure and impair recovery. Additionally, AH_2 deficiency might also alter overall antioxidant homeostasis.

MATERIALS AND METHODS

Chemicals. Guinea pig IgG from serum, anti-guinea pig IgG (whole molecule) peroxidase conjugate, and OPD (10 mg tablets) were purchased from the Sigma Chemical Co., St. Louis, MO. Rabbit antiserum to guinea pig albumin, guinea pig serum albumin, and peroxidase-conjugated goat affinity purified antibody to IgG were obtained from the Organon Teknika Corp., Chapel™ Research Products, Durham, NC. All other chemicals were of analytical grade and obtained from commercial sources.

Animals. Thirty-day-old female Hartley guinea pigs (260–330 g) were purchased from Charles River Breeding Laboratories, Kingston, NY, and housed in plastic cages containing pine shavings in temperature- and humidity-controlled (72 ± 2°F, 50 ± 5% relative humidity), American Association for Accreditation of Laboratory Animal Care (AAALAC) approved animal facilities. After 2–5 days, animals were relocated to 0.3 m^3 stainless steel and glass exposure chambers approved by AAALAC for live-in exposure to gases. During quarantine (2–5 days), the guinea pigs were given standard guinea pig chow and water *ad lib*.

Depletion of tissue AH_2. Since guinea pigs are similar to humans, and dissimilar to most other laboratory animals in terms of their dietary requirement of AH_2, they can be made AH_2 deficient by eliminating AH_2 from the diet. It has been established previously that the rabbit chow (Prolab Animal Diet, Agway Inc., Syracuse, NY), which does not contain added AH_2, can be used successfully to make guinea pigs AH_2 deficient [29]. HPLC analyses of AH_2 in the guinea pig (Conventional Prolab Guinea Pig Diet, Agway Inc., Syracuse, NY) and rabbit chow (Conventional Prolab Rabbit Diet, Agway Inc., Syracuse, NY) indicated that rabbit chow was deficient in AH_2 (0.001 g/kg diet) relative to the guinea pig chow (2.34 g/kg diet). α-Tocopherol levels in both diets were similar as per the information provided by the source company and as analyzed previously in our laboratory [29]. All other respective dietary constituents were of similar composition and concentration, except for total fat and vitamin A, which were lower in rabbit chow when compared with guinea pig chow. Both diets were stored at 4°. In a preliminary experiment, the daily food consumption was monitored to be 20–30 g/animal, and it was suspected that AH_2 in the food left in the exposure chambers (which operated 23 hr/day) may be oxidized in the presence of O_3. To minimize this problem, only 40–50 g of fresh diet pellets were placed daily in the cage feeders, and the residual food was removed the following day. Both chows were consumed equally well. The feeding regimen was started 1 week prior to exposure, and was continued throughout the exposure and 1 week post-exposure recovery. A single diet lot manufactured on the same milling date was used throughout the study.

O_3 exposure. During exposure, the guinea pigs were housed individually in stainless steel cages at a maximum of 8 per chamber on a single tier. Preliminary experiments indicated that housing of guinea pigs in wire mesh cages in the exposure chambers resulted in a temporary reduction in body weight gain. Thus, the guinea pigs were allowed to acclimatize in the chamber for 1 week prior to the exposure (at the time when the dietary regimen was started). After that week, AH_2 levels were measured in ear tissue punches from all guinea pigs. Forty-eight guinea pigs maintained on normal diet and 48 maintained on AH_2-deficient diet were then exposed to 0 (N = 10), 0.2 (N = 4), 0.4 (N = 4) or 0.8 ppm O_3 (N = 6), 23 hr/day for 1 week. One group of the same number of guinea pigs was allowed 7 days of air recovery after the exposure. These concentrations of O_3 were selected based on acute studies so as to approximate minimal to marked effects in normal guinea pigs. The dietary and exposure protocol is schematized in Fig. 1. Ozone was generated from oxygen using a silent arc discharge O_3 generator (model 3V1, O_3 Research Equipment Co., Phoenix,

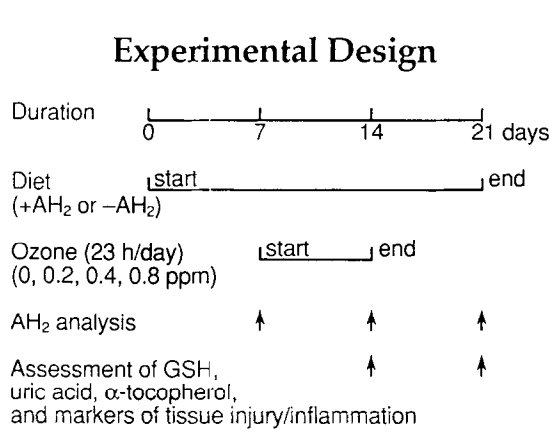

Fig. 1. Experimental protocol for dietary regimen, O_3 exposure and end point analysis. $+AH_2$ = ascorbate (AH_2)-sufficient or normal diet; $-AH_2$ = AH_2-deficient diet.

AZ). The concentrations of O_3 were monitored continuously by an O_3 monitor (model 8002, Combustion Engineering Inc., Lewisburg, WV). The monitors were calibrated against a Dasibi transfer standard that was referenced quarterly to a primary ultraviolet O_3 standard. The concentration range around the target was always less than 3%. The chamber temperature ranged from 69 to 73°F and the relative humidity was 40–60% during preexposure and exposure periods. Immediately after the exposure, half of the guinea pigs were killed and the remaining half were allowed to recover in the animal facility for an additional week.

Lung lavage and cell counts. Guinea pigs were anesthetized with urethane (ethyl carbamate; Sigma), 1 to 1.5 mL/kg body weight, and exsanguinated by severing the abdominal aorta. The left lung was ligated at the main bronchus and the right lung was lavaged *in situ* with saline (37°, 0.85%) [30]. The BAL volume of the right lung was calculated using our preestablished value of 35 mL/kg body weight [30] and the size of the right lung being 62% (determined experimentally) of the total lung weight. The same saline volume was used for three in-and-out washes that were immediately placed on ice. Total cell counts in the lavage were made using a hemocytometer. An aliquot of the lavage was centrifuged using a Shandon Cytospin (Southern Products Ltd., Astmoor, Runcorn, England), the slides were dried at room temperature and stained with Diff-Quick (Fisher Scientific Co., Pittsburgh PA). The remaining lavage was centrifuged at 1500 *g* to remove cells and then divided into aliquots and stored at −80°. One aliquot of cell-free BAL was mixed with an equal volume of 6% perchloric acid, centrifuged at 20,000 *g*, and stored at −80° for AH_2, uric acid and GSH analysis.

Biochemical assays. The apical lobe of the left lung (not lavaged) was used as an index of lung weight and cut into several pieces (about 100 mg each), for subsequent analyses. Small portions of this lung lobe, liver, ear tissue punch, and the adrenal were homogenized (Ultra-Turrex, IKA®-Labortechnik, West Germany, at 70% of the highest speed) in 3% perchloric acid. The homogenates were centrifuged at 4°, 20,000 *g* for 20 min, and stored at −80°. AH_2 and uric acid in the perchloric acid supernatants of BAL and the tissues were analyzed by HPLC (C-18 μBondpack column) using amperometric electrochemical detection [31]. The sample peaks for reduced AH_2 and uric acid were identified based on the peak area of standards. GSH plus glutathione disulfide was measured in perchloric acid supernatants of BAL and tissues by dithionitrobenzene-glutathione disulfide reductase recycling assay [32].

Additional 100-mg tissue samples of apical lobe were homogenized and were extracted in chloroform for total lipid [33]. The chloroform phase was evaporated, and the residue was suspended in cold (−20°) *n*-haptene containing 1.25 mg/100 mL butylated hydroxytoluene. The sample (20 μL) was then injected into an HPLC for analysis of α-tocopherol [34]. α-Tocopherol was separated using a C-18 μBondpack column cartridge, and the signals were detected using a Coulochem electrochemical detector (ESA, model 5100A) set at 0.55 V. All HPLC data were collected and analyzed using a Nelson data system (Nelson Analytical, 760 Series Interface, Cupertino, CA).

The protein content was measured in both BAL and perchloric acid precipitated tissue pellets using a Coomassie Plus Protein Assay kit (Pierce, Rockford, IL). The perchloric acid pellets were first solubilized in 2 M sodium hydroxide and diluted 10–30 times for the analysis. Albumin and IgG were quantified in BAL using non-equilibrium competitive ELISA as described by Rennard *et al.* [35], with modifications. Briefly, polystyrene microtiter immunoassay plates (Immunol 2 'U' bottom, Dynatech Laboratories, Chantilly, VA) were coated with a fixed amount of antigen (guinea pig IgG or guinea pig albumin) at 4° overnight. Plates were then washed four times with PBS/Tween-20, and in the case of albumin, the plates were blocked with 0.05 mg/mL gelatin and for IgG with 0.5 mg/mL albumin at room temperature for 1 hr. A second set of plates was coated with gelatin or albumin for albumin and IgG assays, respectively. A series of concentrations of standard antigen or samples and fixed amounts of primary antibodies were incubated in the first set of plates for 90 min. An aliquot was transferred from each well to a second set of plates. Incubations were carried out at room temperature for 45 min. Because the primary antibody was linked with peroxidase for IgG, 100 μL (10 μg) OPD was added directly to the plates. In the case of albumin, an additional incubation was done with a peroxidase-linked secondary antibody, and the substrate was then added. After 10 min of incubation, the reaction was stopped with sulfuric acid, the color densities were read at 490 nm on an MR 600 Microplate Reader (Dynatech Instruments, Inc., Torrance, CA). The standard inhibition curve was established to calculate the unknown sample values.

Statistics. Because of concerns about using protein as a denominator in edematous lungs, antioxidant values in the lung were expressed per apical lobe

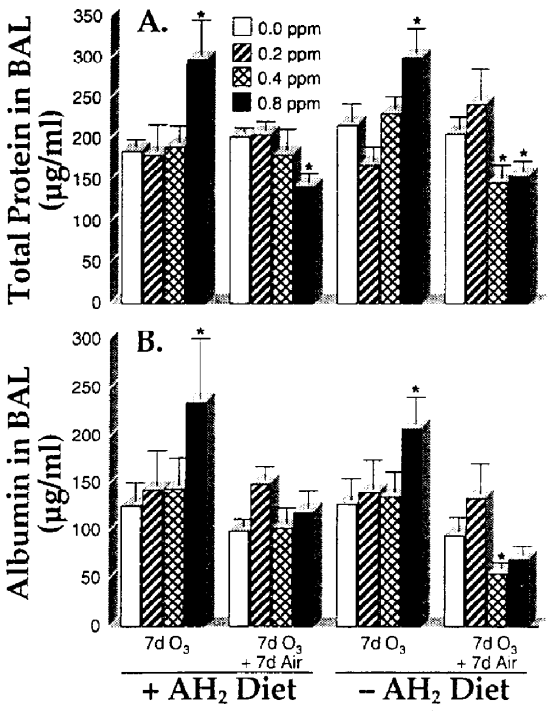

Fig. 2. Total protein (A) and albumin (B) in BAL after 1 week of O_3 exposure and air recovery in normal and AH_2-deficient guinea pigs. Values are means ± SEM of 10 control or 4–6 exposed guinea pigs. Key: (*) significant ($P < 0.05$) difference from respective air-exposed animals.

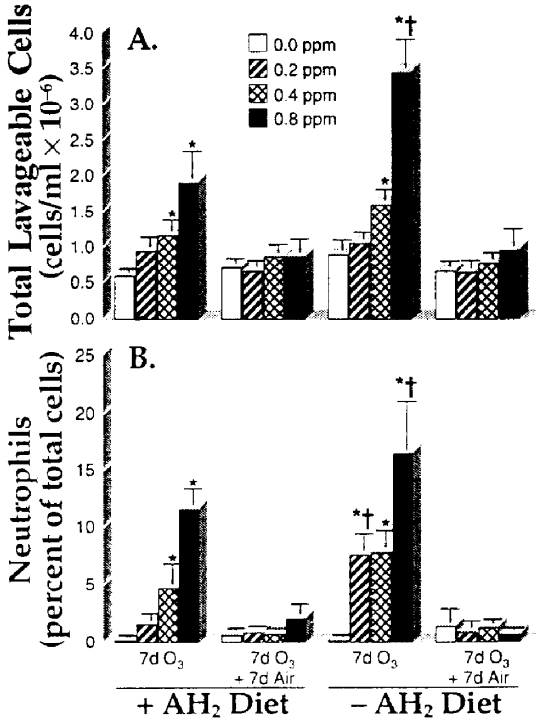

Fig. 3. Total cells (A) and neutrophils (B) in BAL of O_3-exposed normal and AH_2-deficient guinea pigs. Values are means ± SEM of 10 control or 4–6 exposed animals. Key: (*) significant ($P < 0.05$) difference from respective air-exposed animals, and (†) significant ($P < 0.05$) difference from matching AH_2-sufficient animals.

instead of per gram tissue or per milligram protein. Liver and adrenal data, on the other hand, were calculated based on milligram protein; the ear values were expressed per milligram tissue. A MANOVA (SAS 516, SAS Institute, Cary, NC) was used twice to evaluate overall main and interactive effects to help avoid Type I errors (false positives) associated with making multiple univariate comparisons. Each analysis modeled exposure at four levels (0.0, 0.2, 0.4, 0.8 ppm O_3) as one factor and diet at two levels (normal vs AH_2-deficient) as the second factor. The first MANOVA was used to examine effects immediately post-exposure. The second MANOVA was used to examine effects related to the 7-day post-exposure filtered-air period. According to the study protocol, if significant ($P \leq 0.05$) multivariate diet, exposure, or exposure and diet interaction effects were found, further univariate analyses using the same analysis model as used in the MANOVA were performed. Corrected pair-wise comparisons were used to evaluate differences between exposure and diet groups or to examine differences in exposure groups with different diets in the event that significant exposure and diet interactions occurred. The type I error rate was set at $P = 0.05$. Data are expressed as means ± SEM. If significant ($P < 0.05$) overall exposure-only effects were found, then only the asterisk was placed on the bars to show individual comparisons. Significant diet and exposure interactions are indicated as a single dagger.

RESULTS

Lung injury and inflammation. BAL protein, albumin, IgG, total cells and differentials were analyzed as putative markers of lung injury/inflammation. The MANOVA derived from BAL protein and albumin indicated that there were no significant exposure and diet interactions; however, significant O_3 effects were seen (BAL protein, $P = 0.0085$; albumin, $P = 0.022$). An increase in total BAL protein and albumin was apparent immediately after the exposure at 0.8 ppm O_3 in both normal and AH_2-deficient guinea pigs (Fig. 2, A and B). AH_2 deficiency did not have any influence on O_3-induced increase in BAL protein and albumin. After 1 week of air recovery, protein (at 0.8 ppm in normal; at 0.4 and 0.8 ppm in AH_2-deficient guinea pigs) and albumin levels (at 0.4 ppm in AH_2-deficient guinea pigs) in the BAL fell below control value. BAL IgG was not altered at any time after O_3 exposure in either dietary group (data not shown).

A small but significant exposure and diet interaction ($P = 0.018$) was observed in total cells in lavage (Fig. 3A). Total cells in lavage increased in a concentration-dependent manner in normal as well as AH_2-deficient guinea pigs following O_3 exposure (Fig. 3A). The magnitude of increase at 0.8 ppm O_3 was slightly greater in AH_2-deficient when compared

Table 1. Systemic ascorbate (AH_2) depletion in guinea pigs fed an AH_2-deficient diet

Dietary regimen (days)	% AH_2 remaining				
	Lung	BAL	Liver	Ear	Adrenal
7	29.7 ± 4.1	10.5 ± 3.9	17.1 ± 5.0	20.7 ± 2.4	21.6 ± 4.0
14	1.2 ± 0.9	6.0 ± 3.9	4.0 ± 2.4	2.5 ± 0.5	6.9 ± 0.8
21	1.0 ± 0.5	4.6 ± 2.1	1.6 ± 0.5	0.5 ± 0.1	1.1 ± 0.2

AH_2 levels in the tissues of guinea pigs fed an AH_2-sufficient diet: lung, 28.7 ± 7.3 nmol/mg protein (N = 10); BAL, 6.2 ± 1.6 nmol/mL (N = 10); liver, 14.4 ± 5.7 nmol/mg protein (N = 10); ear, 0.45 ± 0.05 nmol/mg tissue (N = 21); and adrenal, 111.4 ± 6.1 nmol/mg protein (N = 10). Values in the table represent means ± SEM of percent AH_2 remaining.

with AH_2-sufficient guinea pigs. Neutrophils were also increased in an O_3 concentration-dependent manner in both dietary groups, and the magnitude of increase was slightly greater in AH_2-deficient guinea pigs at 0.2 and 0.8 ppm O_3. The increase in total cell numbers reflected the overall increase in macrophages (data not shown) and neutrophils. Eosinophils and ciliated or other unidentified cells (data not shown) did not change significantly with diet or O_3. The unidentified cells, perhaps lymphocytes or monocytes, were always less than 5% of the total cells.

The O_3-induced changes in total cells and neutrophils observed immediately after exposure diminished to nearly control levels after 1 week of clean air recovery in both normal and AH_2-deficient guinea pigs (Fig. 3, A and B).

Ascorbic acid. AH_2 was measured in BAL and several tissues in order to understand its compartmentalization and homeostasis (Table 1). Analysis of AH_2 in ear tissues of the AH_2-deprived animals taken just prior to the beginning of exposure (1 week on the AH_2-deficient diet) indicated a 79% drop. This fall in ear AH_2 was comparable to the AH_2 decrease in lung, liver and adrenal gland. At this time point, BAL AH_2 showed an even greater depletion (~90%). With 2 and 3 weeks of AH_2-deficient diet, all tissues and BAL in the air-exposed guinea pigs were severely (>93%) depleted of AH_2 (Table 1). MANOVA indicated a significant exposure and diet interaction in BAL ($P = 0.001$) and lung tissue AH_2 ($P = 0.001$). In normal guinea pigs, O_3 exposure at all levels caused increases in BAL (Fig. 4A) and lung tissue AH_2 (Fig. 4B). In the AH_2-deficient guinea pigs, a trend of O_3-induced increase in BAL and lung AH_2 was noticeable immediately after the exposure; however, this change was not significant. The alterations in BAL and lung tissue AH_2 in AH_2-sufficient guinea pigs were reversed only partially following 1 week of recovery (Fig. 4, A and B). Liver levels of AH_2 (Fig. 4C) appeared to be increased at low O_3 concentrations and diminished at 0.8 ppm with no defined trend of concentration-related changes. Ear and adrenal gland AH_2 levels did not vary significantly in response to O_3 either at the end of exposure or 1 week post-exposure (data for exposed guinea pigs are not shown).

Uric acid. AH_2 deficiency did not change the basal levels of uric acid in any of the tissues (Fig. 5). BAL and lung uric acid in normal AH_2 animals increased, reaching significance at 0.8 ppm (Fig. 5, A and B). The AH_2-deficient guinea pigs appeared to have a slightly exaggerated O_3-related increase relative to normal animals. There was wide variation in liver uric acid especially in the guinea pigs killed immediately after the exposure (Fig. 5C). Adrenal gland uric acid was also widely variable, but was unchanged in response to O_3 or diet (data not shown). The changes observed in the BAL and lung tissue were reversed at the end of the 1-week clean air recovery period. Higher BAL uric acid level at 0.2 ppm in the AH_2-deficient recovery group may reflect overall variability in the values obtained in the analysis.

GSH. BAL, lung and liver GSH were not affected by severe AH_2 deficiency in air-exposed animals (Fig. 6). GSH in the BAL and lung tissue, on the other hand, was increased in response to O_3 at 0.8 ppm ($P = 0.0017$ for BAL and $P = 0.0001$ for lung tissue) in both dietary groups. AH_2 deficiency had no significant impact on the O_3-induced elevation in lung ($P = 0.94$) or BAL GSH ($P = 0.75$) (Fig. 6, A and B). GSH levels in the liver (Fig. 6C) and adrenals (data not shown) remained unaltered by dietary regimen or by O_3. At the end of 1 week of air recovery, the GSH increases observed in BAL, but not in lung tissue, were reversed almost entirely (Fig. 6, A and B).

α-Tocopherol. Unlike GSH and uric acid, there was an overall diet effect on lung tissue α-tocopherol levels ($P = 0.004$). AH_2 deficiency decreased α-tocopherol levels irrespective of O_3 or recovery status (Fig. 7). O_3 exposure did not change lung α-tocopherol significantly (Fig. 7).

DISCUSSION

O_3 increased BAL proteins and caused an inflammation in a concentration-dependent manner in the normal guinea pig lung, which is consistent with the reported findings in the literature (1–6). Since O_3 toxicity from acute exposure (4 hr) is enhanced by AH_2 deficiency as measured in BAL protein [27], we anticipated that long-term O_3 might have a more severe effect on BAL protein and inflammation in AH_2-deficient guinea pigs. However, unlike in the acute (4 hr) O_3 exposure study [27],

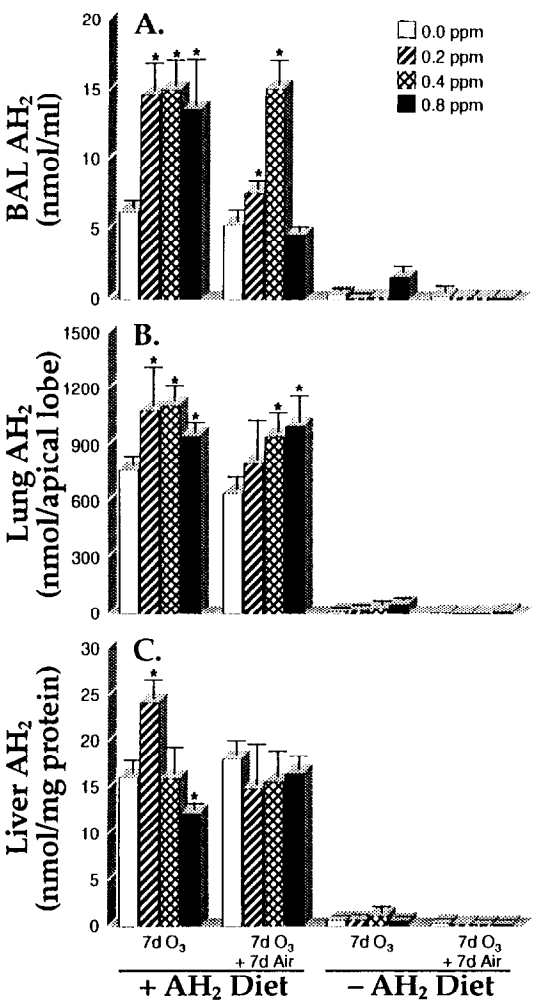

Fig. 4. O_3 exposure and AH_2 deficiency-related changes in AH_2 levels in BAL (A), lung (B) and liver (C). The lung analysis was done with a small portion of the tissue collected from the apical lobe, and the values were expressed per apical lobe. The liver values are expressed per milligram protein. Each bar represents the mean ± SEM of 10 control or 4–6 exposed guinea pigs. Key: (*) significant ($P < 0.05$) difference from respective air-exposed animals. AH_2 values of BAL, lung and liver tissues in all $-AH_2$ groups were always significantly ($P < 0.0001$) lower than $+AH_2$ groups (symbol for significant difference is not given in the figure for clarity).

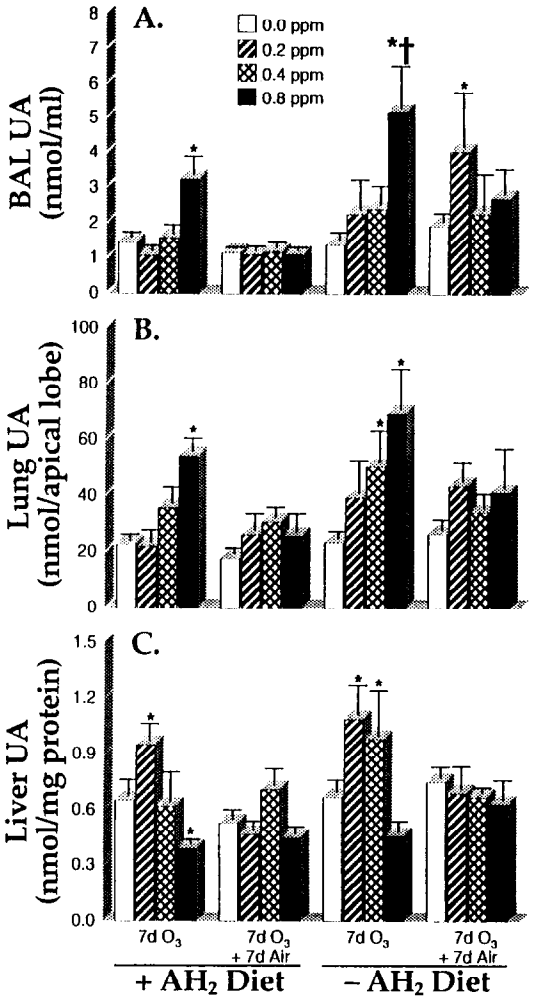

Fig. 5. Uric acid in the BAL (A), lung (B) and liver (C) of O_3-exposed normal and AH_2-deficient guinea pigs. Each data point represents the mean ± SEM of 10 control or 4–6 exposed animals. No overall exposure-related differences were seen in liver, although the values in different groups were variable with no specific patterns of change. Key: (*) significant ($P < 0.05$) difference from respective air-exposed animals, and (†) significant ($P < 0.05$) difference from matching AH_2-sufficient animals.

AH_2 deficiency only marginally exacerbated O_3 toxicity, as measured by BAL proteins and cellular influx. In a companion study, we have also reported that O_3-induced lung function abnormalities and histopathology after 1 week of continuous exposure were only exaggerated marginally by AH_2 deficiency in guinea pigs [28]. These data support the notion that although AH_2 is probably involved in functional adaptation [8] and plays a protective role in exposures of short (4 hr) duration [27], it is not critical to the process of repair/adaptation in the continuous 1-week exposure regimen in guinea pigs.

In the present study, we addressed two issues related to the antioxidant functions of AH_2, which we had hypothesized as important to host defense against O_3. First, by inducing systemic AH_2 deficiency, we asked whether the profile of the other antioxidants in the lung and other tissues would be altered. Second, we sought to know how O_3 would influence these antioxidants and if there was any relationship between changes in the antioxidants and resulting O_3 toxicity. From this study, it was clear that severe AH_2 deficiency did not have a significant impact on base-line tissue uric acid or GSH levels in air-exposed guinea pigs. The slight depression of α-tocopherol due to AH_2 deficiency, however, is consistent with the suggested role for AH_2 in

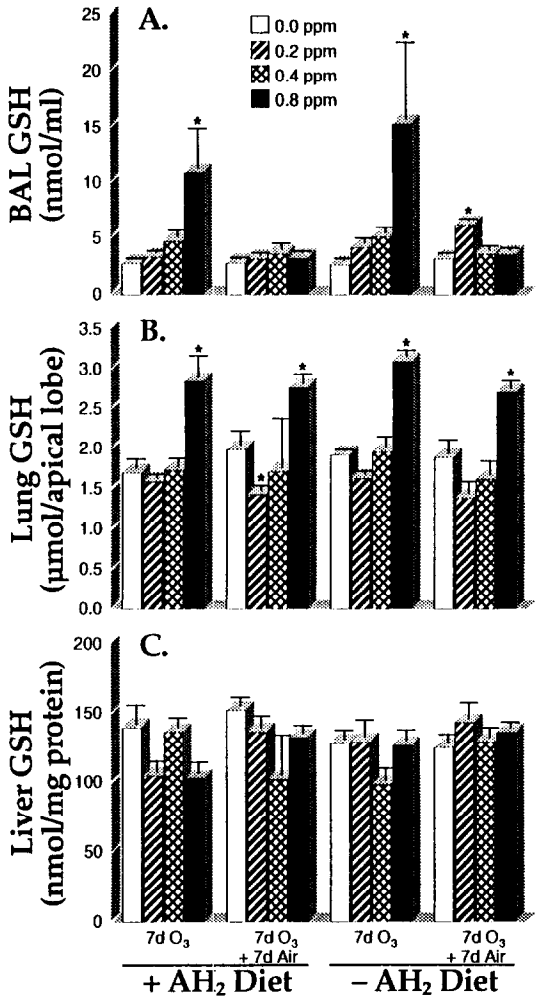

Fig. 6. O_3-induced changes in the BAL (A), lung (B) and liver (C) GSH in normal or AH_2-deficient guinea pigs. Each bar represents the mean ± SEM of 10 control or 4–6 exposed guinea pigs. Adrenal tissue was also analyzed but showed no exposure or diet-related changes (data not given). Key: (*) significant ($P < 0.05$) difference from respective air-exposed animals.

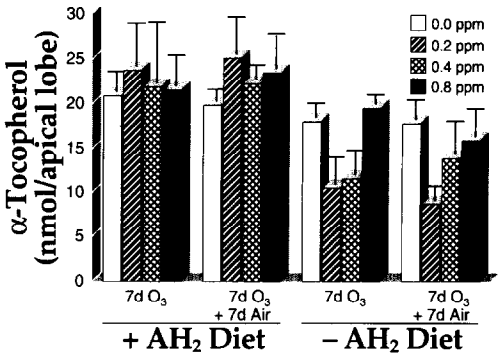

Fig. 7. Effect of O_3 exposure on α-tocopherol levels in the lungs of normal and AH_2-deficient guinea pigs. Values are means ± SEM of 10 control or 4–6 exposed guinea pigs. Significant overall diet-only effects were seen at both time points (immediately after exposure, $P = 0.004$; 1 week post-exposure, $P = 0.0002$).

protecting α-tocopherol from oxidation [19, 36]. The maintenance of uric acid and GSH in the reduced form, on the other hand, is less likely to be dependent upon the availability of AH_2 in the tissues [37, 38]. Thus, in the absence of O_3, AH_2 deficiency does not appear to influence the homeostasis of GSH and uric acid significantly.

Induction of antioxidant substances and enzymes has been reported after O_3 exposure in several animal species [11, 12, 39]. Consistent with the induction of antioxidant mechanisms, we observed increases in lung AH_2, uric acid and GSH after 1 week of continuous O_3 exposure. The increase in lung and BAL AH_2 after O_3 exposure in normal guinea pigs was probably due to increased uptake from the plasma, which would, in turn, be reflected in the liver and other tissue storage sites. In the mouse, it has been observed that AH_2 can be mobilized from the liver to the lung during O_3 exposure [40, 41]. Since tissue mass and AH_2 concentrations are higher in the liver than the lung or epithelial lining fluid, mobilization to the lung should not greatly affect liver AH_2 levels as was noticed.

Uric acid appears to be a major airway antioxidant in human nasal secretions [22], though much less is found in rodents [42]. Increases in BAL uric acid have been observed previously with short-term O_3 exposure [42]. Our results indicate that longer-term O_3 exposure can also induce uric acid accumulation, not only in BAL but also in the lung tissue. Plasma uric acid is high, and O_3-induced lung injury may cause plasma leakage and subsequent uric acid leakage into the airspaces, which could be one of the plausible explanations. Alternatively, O_3 exposure could increase the xanthine oxidase pathway of adenosine metabolism, which might cause uric acid to accumulate [43].

Induction of GSH and its related enzyme systems after O_3 exposure has been reported in several studies [12, 39]. We also observed an increase in BAL and lung GSH regardless of diet and it remained elevated in the lung during the recovery period, suggesting its probable role in adaptive or reparative processes especially at higher concentrations (0.8 ppm). The increase of GSH was clearly evident at 0.8 ppm, but not at 0.4 ppm. Ichinose and Sagai [44] have reported that O_3 exposure does not increase GSH in guinea pig lungs following continuous exposure to O_3 at 0.4 ppm. AH_2 deficiency appears to have no influence on O_3-induced increases in BAL and lung GSH. This could be due to the fact that cellular GSH is maintained in the reduced form by a major reductase pathway that does not involve AH_2 [38].

α-Tocopherol, unlike other antioxidants, was not increased by O_3 exposure in AH_2-sufficient animals. The slight depletion in lung α-tocopherol due to AH_2-deficient diet, at both time points and regardless

of O_3, could be due to insufficient reduction of α-tocopherol radicals by AH_2 [19, 36]. Moreover, the lack of increase in α-tocopherol levels after O_3 exposure is consistent with previous studies [45].

The changes in BAL inflammatory markers induced by O_3 in the normal and in the AH_2-deficient groups were only apparent immediately after exposure and were resolved almost entirely after 1 week post-exposure in air. The lack of effect of O_3 on another marker of lung inflammation, IgG (immediately after the exposure and recovery), in both dietary groups may indicate attenuated permeability response upon 7 days continuous exposure, regardless of the diet. Other studies have reported that IgG in the BAL returns to control levels after 1 week of continuous O_3 exposure of normal mice and dogs after the early increases on days 1–3 [46, 47]. In a related study, we observed almost complete reversal of O_3-induced histopathological lesions and functional abnormalities 1 week post-exposure, irrespective of AH_2 status [28]. This may suggest either an adaptation of the lung not involving AH_2 or a role for other antioxidants in adaptation and repair (such as GSH, which remained elevated even after 1 week post-exposure). The role of AH_2 in repair remains unclear: either it is not as important as hypothesized or its intrapulmonary level or distribution was sufficient to maintain focal repair.

One of the ancillary objectives of this study was to monitor the deficiency status of each animal using a minimally invasive method. It was observed with our preliminary experiment that the analysis of plasma AH_2 was variable and that blood sampling from the guinea pig was not an easy procedure. Since the assay for AH_2 is very sensitive and requires only a small amount of sample, we wanted to see if AH_2 levels could be monitored from ear tissue punches (10–25 mg). Ear tissue AH_2 analysis revealed a diet-dependent drop in AH_2 comparable to that of other body tissues, and the punched ear tissues healed quickly without bleeding or infection. The AH_2 values were reproducible and consistent within the same group of animals. The success of this effort suggests that measurements of other biochemical indices in ear tissue may be useful for monitoring the nutritional status in animals.

In summary, the results support the following conclusions: (1) AH_2 deficiency without O_3 exposure does not alter the homeostasis of GSH and uric acid significantly; (2) AH_2 deficiency only slightly enhances lung injury/inflammation induced by 1 week of O_3 exposure; (3) recovery from O_3-induced cell injury is nearly complete and is not affected by AH_2 deficiency, suggesting that reparative processes are not altered by systemic AH_2 deficiency; (4) antioxidants appear to increase in response to O_3 exposure despite the deficiency in AH_2; and (5) monitoring of AH_2 deficiency status could be accomplished effectively using ear tissue punches.

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