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Uric acid is a major antioxidant in human nasal airway secretions

(respiratory tract/submucosal gland)

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ABSTRACT **Airway mucosal surfaces are potentially subjected to a variety of oxidant stresses. Airway submucosal glands secrete a variety of compounds that may protect the airways from injury. Cholinergically induced nasal submucosal gland secretion has recently been found to contain a low molecular weight nasal antioxidant. In this report, the isolation and identification of this nasal secretory antioxidant are described. Concentrated, cholinergically induced human nasal secretions were fractionated through a 10-kDa sieve and subjected to DEAE anion-exchange chromatography. Fractions containing antioxidant activity were subjected to gel filtration with Bio-Gel P-2 gel (resolution range, 200–2000 Da). The resultant antioxidant fractions were then desalted by gel filtration over the same column equilibrated in HPLC-grade water, yielding only a single peak with antioxidant activity. The absorption spectrum of the purified antioxidant revealed peaks at 238 and 292 nm at pH 7. These peaks shifted to 230 and 280 nm in 0.1 M HCl and to 226 and 296 nm in 0.1 M NaOH. Sodium borohydride reduction of the antioxidant had no effect on the UV absorption, whereas platinum-catalyzed hydrogenation ablated all absorption peaks. Uric acid had identical absorption peaks and showed the same chromatographic behavior as the nasal antioxidant activity on both gel filtration and DEAE columns. Uricase (which degrades uric acid) metabolized both uric acid and the purified antioxidant. Uric acid was shown to have antioxidant activity at concentrations >1.5 μM. These data indicate that nasal secretions contain uric acid that serves as an antioxidant.**

Exposure to excessively high oxygen tension plays a pathogenic role in both the adult respiratory distress syndrome (1, 2) and the development of bronchopulmonary dysplasia in preterm neonates (3). Ozone exposure has also been shown to adversely affect lung function, both in normal volunteers and in asthmatics (4, 5). Active oxygen species generated by inflammatory cells may be cytotoxic both to microorganisms and to host tissues (6, 7). Antioxidants in tissue fluids act to attenuate the effects of increased oxygen *in vivo* and *in vitro*. Naturally occurring antioxidants include proteins such as superoxide dismutase, catalase, and transferrin, and small molecules such as glutathione, ascorbic acid, and vitamin E. Each of these antioxidants has been found in either pulmonary epithelial lining fluid or within pulmonary tissues (8–13).

Nasal submucosal glands secrete a variety of molecules that participate in host defense and in maintenance of mucosal homeostasis. These molecules include lactoferrin, lysozyme, secretory IgA, and mucous glycoproteins (14–18). Glandular secretions are stimulated by cholinergic (14), peptinergic (18), and allergic or reflex (17) actions. Nasal submucosal glands are morphologically and functionally similar to glands found in the trachea and bronchi. Thus the study of nasal glandular function may serve as a model for the study of lower airway glandular function as well.

Some premature infants are predisposed to the development of bronchopulmonary dysplasia (BPD), an airway disorder caused by exposure to enriched oxygen. Those infants who develop BPD have recently been found to be relatively deficient in airway lactoferrin and lysozyme, suggesting that submucosal gland prematurity may predispose to the disease (19). This observation led to the search for the presence of airway submucosal glandular antioxidants in secretions. An antioxidant secreted in response to cholinergic stimulation was found in human nasal secretions and this factor was partially characterized. The nasal secretory antioxidant was found to have a molecular weight less than 1000, to be separable from all other known antioxidants, and to be secreted under cholinergic control along with the serous cell products of submucosal glands (20). In the current study, purification of the nasal antioxidant revealed that it had sizing, spectral, and reductive characteristics consistent with uric acid. Indeed, exogenous uric acid cofractionated with the antioxidant activity in nasal secretions and both molecules were oxidized by uricase, an enzyme specific for uric acid metabolism.

METHODS

Subjects. Subjects studied in these experiments were normal, healthy volunteers with no history of rhinitis or sinusitis for at least 6 months. These subjects were nonsmokers, had no recent symptoms of viral or bacterial upper respiratory tract infections, and had normal appearing nasal mucosa by physical examination prior to study. Each subject gave informed consent and the protocol was approved by the National Institutes of Health institutional review board. A total of 18 subjects underwent 40 challenge sessions.

Nasal Challenge Technique. The provocation and collection techniques have been described in detail (14). Each study was done employing both nares. Before each challenge, the nasal cavities were rinsed by spraying 4 ml of 0.9% NaCl into the nares with a hand-held nebulizer. After these prechallenge rinses were performed, each patient underwent four sequential methacholine (25 mg) challenges. Ten minutes after each challenge, the nasal cavities were rinsed with sterile water. Challenge samples were stored in ice until the challenge session was complete, at which time they were pooled with samples from five other individuals and stored at −70°C. Previous studies have revealed that nasal washings collected by this procedure are acellular, and therefore it was unnecessary to centrifuge the samples prior to storage. With this procedure, 85% of administered rinse solution is recovered (14).

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Abbreviations: BPD, bronchopulmonary dysplasia; HRP, horseradish peroxidase.

Preparation of Nasal Secretion Concentrates. The nasal washings were lyophilized and resuspended in phosphate-buffered saline (PBS; Biofluids, Rockville, MD) at 1/40th of the original volume. The concentrates were centrifuged at 20,000 × *g* for 30 min, yielding a mucus pellet and clear supernatant. The supernatants were saved for fractionation and the pellets were discarded. Initial fractionation of the concentrates was performed by centrifugation through a 10-kDa cutoff Centricon microconcentrator (Amicon). The filtrates were collected and concentrated by lyophilization to 200 times the original concentration prior to storage at −70°C.

Additionally, nasal secretions collected from a group of six subjects were examined individually to determine patient-to-patient variability in antioxidant response. After prechallenge rinses were completed, sequential challenges using 0.9% NaCl and 25 mg of methacholine were performed at 10-minute intervals. Sample collection was performed similarly to that described above except that saline was used instead of sterile water. These samples were stored individually and were not concentrated. Fractionation of individual collections through 10-kDa filters was performed as described. Each sample was then analyzed for antioxidant activity with a chemiluminescence assay (see below) and compared to a dose–response curve of the antioxidant activity of uric acid. The antioxidant activity of the secretions was expressed as the micromolar dose of uric acid that had equivalent antioxidant activity (uric acid equivalents). Samples were stored at −70°C until analysis. Statistical significance was determined using the Wilcoxon signed rank test.

Horseradish Peroxidase (HRP) Chemiluminescence Assay. A chemiluminescence assay was used to determine the antioxidant activity of the preparations. HRP was dissolved at 0.3 μg/ml in PBS (pH 7.5). Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione; Sigma) was dissolved in 500 μl of dimethyl sulfoxide. PBS was added until the luminol concentration was 0.1 mM. Fifty microliters each of HRP solution, luminol solution, and sample were mixed in a 250-μl well of an ELISA plate. The reaction was initiated by adding 50 μl of 2 mM H_2O_2 and the contents were immediately transferred into a small liquid scintillation counting vial (Ponyvial, Packard Instrument) and the resultant chemiluminescence was measured in a Packard 2200CA liquid scintillation counter set on single-photon mode. Chemiluminescence was recorded as cpm. A reference reaction employing PBS instead of nasal secretions was run with each group of samples. The percent inhibition of a given sample was determined as 100 − [(sample cpm/reference cpm) × 100].

Anion-Exchange Chromatography. Five hundred microliters of concentrated filtrates was fractionated by anion-exchange chromatography on a DEAE-5PW column (7.5 cm; Tosohaas, Philadelphia) equilibrated with 20 mM phosphate buffer (pH 7). The column was developed with a linear gradient of KCl (0–700 mM) in 35 min at a flow rate of 0.50 ml/min. The fractions were monitored by A_{210} and A_{280}. All fractions were assayed for antioxidant activity with the HRP chemiluminescence assay. The fractions with antioxidant activity were pooled, lyophilized, and dissolved in 500 μl of 50 mM phosphate buffer (pH 7) in preparation for the next isolation procedure.

Gel Filtration Chromatography. Two hundred microliters of the fractions containing antioxidant activity from the previous step was subjected to gel filtration through a 1 × 25-cm column packed with Bio-Gel P-2 (Bio-Rad), which resolves molecules ranging from 200 to 2000 Da. This column was equilibrated in 50 mM phosphate buffer and the flow rate was 0.15 ml/min. Each fraction was assayed for antioxidant activity. Fractions with antioxidant activity were pooled, lyophilized, and dissolved in 500 μl of HPLC-grade water. To desalt the active fractions, this preparation was then resubjected to filtration over the same Bio-Gel P-2 column, after the column had been reequilibrated in HPLC-grade water.

Antioxidant Identification. The UV absorbance characteristics of the purified antioxidant were analyzed with a Hewlett–Packard 8452A diode-array spectrophotometer by diluting 50 μl of antioxidant into 450 μl of either PBS, 0.1 M HCl, or 0.1 M NaOH. The purified antioxidant was subjected to reductive conditions by adding 1 mg of sodium borohydride to the cuvette. A second reduction, employing 1 mg of platinum oxide and hydrogen, was performed on a separate aliquot in a similar cuvette.

The ability of uricase (which enzymatically metabolizes uric acid to allantoin) to degrade the purified nasal antioxidant was examined by placing 50 μl of either the antioxidant or uric acid (diluted to the same concentration as judged by A_{292}) into 450 μl of 0.7 M glycine buffer (pH 9.4) and adding 10 μl of uricase (1 unit/ml; Sigma) and allowing the reaction to proceed for 10 min at 25°C. UV absorption spectra of these samples were obtained before and after addition of uricase. A uricase blank was subtracted from determinations of samples containing this enzyme.

RESULTS

Chromatographic Isolation. DEAE anion-exchange chromatography of a concentrated low molecular weight preparation of nasal secretions yielded six adjacent fractions with antioxidant activity (96–99% inhibition of the reference peroxidase reaction) that were eluted from the column after 15–17 min (Fig. 1*A*). The active fractions were pooled, concentrated to 200 μl, and subjected to gel filtration on Bio-Gel P-2 in 50 mM phosphate buffer. This procedure yielded four adjacent fractions with antioxidant activity (96–99% inhibition of the reference peroxidase reaction) that were eluted from the column at 175–190 min, whereas no other fractions had antioxidant activity (Fig. 2*A*). The elution pattern of the antioxidant activity from the P-2 sizing column

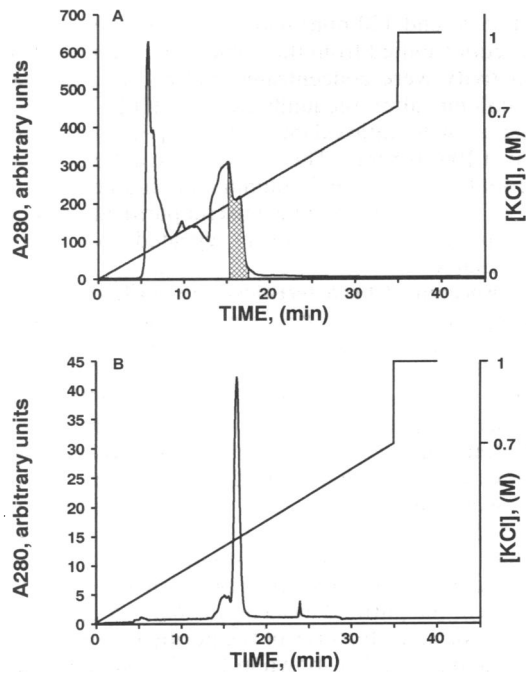

FIG. 1. DEAE anion-exchange HPLC. (*A*) Chromatogram of nasal secretory antioxidant activity eluted with a 0–700 mM KCl gradient in 20 mM phosphate buffer (pH 7). Fraction size was 0.4 ml; flow rate was 0.5 ml/min. Activity was eluted at 15–18 min (cross-hatched area). (*B*) Elution of 15 mM uric acid under similar conditions. Elution time was 16 min (identical to that of the antioxidant in *A*).

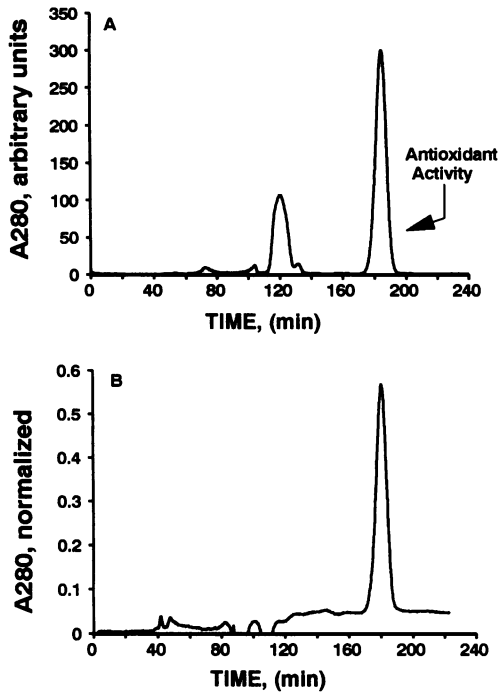

FIG. 2. Elution of partially purified nasal antioxidant activity or of uric acid from a 1 × 30-cm Bio-Gel P-2 column in 50 mM phosphate buffer (pH 7) at a flow rate of 0.15 ml/min. (*A*) Nasal antioxidant activity was eluted at 175–190 min. (*B*) Uric acid (15 mM) was eluted at 180 min. Ordinate represents A_{280} as arbitrary units (*A*) or normalized such that each 0.1-unit change represents 10% of the total absorbance signal monitored from 0 to 240 min (*B*).

suggested that this activity resided in molecules of <500 Da. Exogenous ascorbic acid and glutathione (two antioxidants previously described in respiratory secretions) were also run on this column and eluted at 90 and 120 min, respectively. Corresponding fractions from the nasal secretion sample (eluted at 90 and 120 min) had no antioxidant activity. The four fractions eluted from the column that contained antioxidant activity were concentrated and desalted on the same sizing column after reequilibration in HPLC-grade water, yielding a single antioxidant peak that was eluted from the column at 100–108 min. The difference in antioxidant elution time from the Bio-Gel P-2 column equilibrated in phosphate buffer vs. water proved to be a useful purification step. This three-step procedure has been repeated five times with similar results.

UV Absorbance Characteristics. The purified nasal antioxidant preparation in PBS (pH 7) had absorbance peaks at 292 and 238 nm (Fig. 3*A*). These peaks shifted to 280 and 230 nm in 0.1 M HCl and to 296 and 226 nm in 0.1 M NaOH. Exposure to sodium borohydride had no effect on the UV absorption spectrum of the purified antioxidant. Catalytic hydrogenation with platinum caused all absorption peaks to decrease.

Review of these characteristics with those of known, water-soluble antioxidants with molecular sizes <500 Da revealed that uric acid had very similar UV characteristics. Repeating the UV absorbance studies with uric acid confirmed that its UV absorbance characteristics were identical with those of the purified nasal antioxidant. A comparison of the UV absorbance features of the purified nasal antioxidant and uric acid at pH 7 is shown in Fig. 3. Uric acid exhibited the same spectral shifts in acid and base as did the purified nasal antioxidant.

Exogenous uric acid was chromatographed on the DEAE (Fig. 1*B*) and the gel filtration (Fig. 2*B*) columns and found to fractionate with precisely the same pattern as the nasal secretory antioxidant activity.

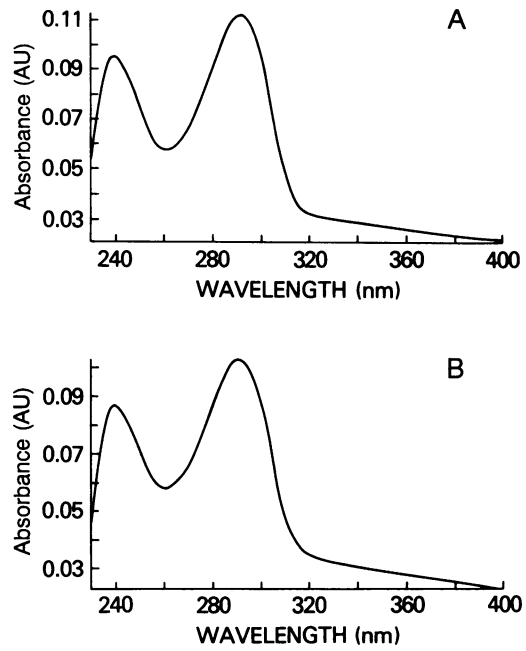

FIG. 3. UV absorption spectra of the purified nasal antioxidant preparation (*A*) and uric acid (*B*) in PBS at pH 7. The spectrum of the purified nasal antioxidant has absorbance maxima at 238 and 292 nm. AU, absorbance units.

Effects of Uricase on the Antioxidant Activity. Uric acid is metabolized to allantoin by the action of uricase. To confirm our suspicions that uric acid might be the nasal secretory antioxidant, both exogenous uric acid and a purified prepa-

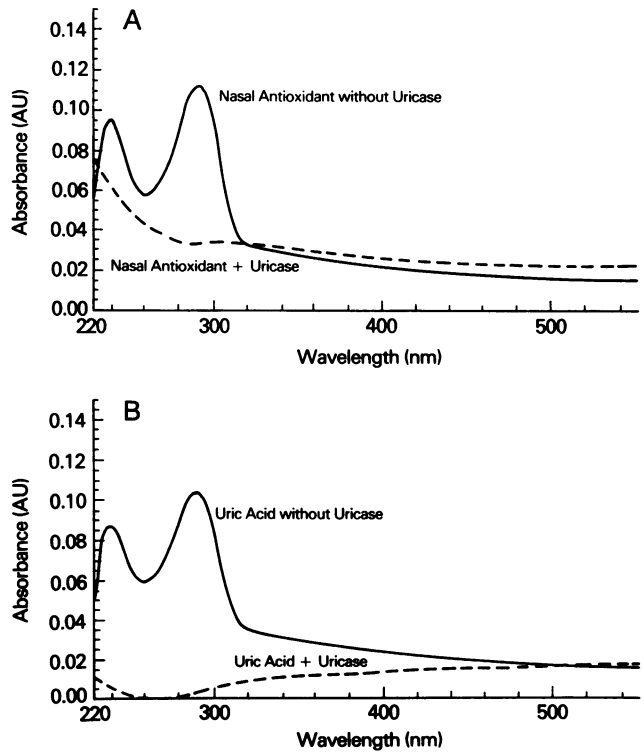

FIG. 4. Effect of uricase on the purified antioxidant (*A*) and uric acid (*B*). Fifty microliters of uric acid or antioxidant preparation was placed into 450 μl of 0.7 M glycine buffer (pH 9.4) and the reaction was initiated by the addition of 0.01 unit of uricase. The reaction was performed at 25°C and was complete by 10 min. The UV absorbance spectra are shown before (solid lines) and after (dashed lines) treatment with uricase. AU, absorbance units.

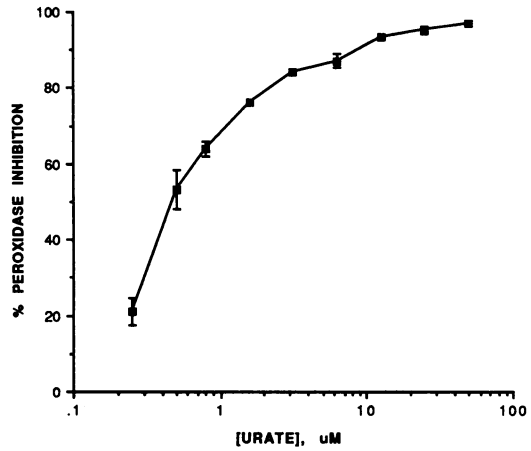

FIG. 5. Dose response of the antioxidant activity of uric acid. Antioxidant activity was determined by the ability of uric acid (urate) to inhibit peroxidase activity. Each dose was tested in duplicate experiments. Mean antioxidant values (% peroxidase inhibition) ± SEM are depicted for each concentration of urate tested.

ration of nasal secretory antioxidant were exposed to uricase for 10 min (Fig. 4). The UV absorption of both uric acid and the nasal antioxidant was eliminated by uricase. These data, taken together with the identical chromatographic behavior of the two molecules, identify the nasal secretory antioxidant as uric acid.

Antioxidant Activity of Uric Acid and Nasal Secretions. Uric acid was found to have antioxidant activity at concentrations as low as 0.25 μM. The antioxidant dose–response curve for uric acid ranged from 20.9 ± 3.6% inhibition at 0.25 μM to 96.9 ± 0.1% inhibition at 50 μM (Fig. 5). The concentration-dependent antioxidant activities of individual nasal secretions (n = 6 subjects) obtained after saline and methacholine challenge are shown in Table 1. The mean antioxidant activity of saline-induced secretions was equivalent to 4.67 ± 1.96 μM uric acid, whereas the antioxidant activity of methacholine-induced secretions was significantly greater, equivalent to 16.06 ± 8.9 μM uric acid ($P \leq 0.014$, n = 6).

DISCUSSION

This report details the isolation of a low molecular weight antioxidant from methacholine-induced human nasal secretions. This antioxidant had UV spectral and chromatographic properties identical to those of uric acid. Additionally, both the purified nasal secretory antioxidant and uric acid were degraded by uricase. These data provide strong evidence that the nasal antioxidant is uric acid. Moreover, these and previously published data (20) indicate that uric acid is the major stable low molecular weight antioxidant present in nasal airway secretions.

Although uric acid has not been previously appreciated as a major respiratory antioxidant, it has been shown to act as an antioxidant in plasma and tissue. In 1981, Ames et al. (21) demonstrated that uric acid, in physiologic plasma concentrations, protected erythrocytes and erythrocyte ghosts from peroxidative stress and reduced the oxidant formed by peroxide interaction with hemoglobin. Uric acid also acts as an antioxidant in guinea pig hearts (22) and is implicated in antioxidant defenses of rat neural tissue (23). The mechanism of action of uric acid as an antioxidant may relate to the formation of a urate free radical after oxidant exposure (24). This free radical can be scavenged by ascorbate, which suggests that urate and ascorbate may interact as plasma antioxidants (24). A review of the literature reveals three recent reports identifying uric acid in nasal and lower respiratory secretions after ozone exposure in guinea pigs, rats,

Table 1. Antioxidant activity of individual nasal secretions

Patient	Uric acid equivalents, μM	
	Saline	Methacholine
1	0.5	2.25
2	1.6	13
3	8	11
4	3.2	6
5	1.7	4.1
6	13	60
Mean ± SEM	4.67 ± 1.96	16.06 ± 8.92 ($P < 0.014$)

Antioxidant activity of nasal secretions from six individual patients. The antioxidant activity from saline- and methacholine-induced nasal secretions from each patient was determined by HRP chemiluminescence inhibition assay (see text). The antioxidant activity was then compared to that of various concentrations of uric acid and was expressed as the micromolar dose of uric acid that gave similar inhibition (uric acid equivalents). Statistical significance was determined using the Wilcoxon signed rank test.

and humans (25–27). These observations, in conjunction with the data presented here, indicate that uric acid may play an important role in airway antioxidant physiology.

Unlike other mammals (except for higher apes and the dalmatian dog), humans do not possess uricase, the enzyme responsible for the metabolism of uric acid. Thus, in humans, uric acid is the end product of purine metabolism. Both uric acid and superoxide are products of the action of xanthine oxidase on xanthine and hypoxanthine (28). Excretion of uric acid occurs primarily in the kidney, although the gastrointestinal tract also generates uric acid (29). While in solution, uric acid (urate) has no significant toxicities. Only when it crystallizes in tissues does uric acid have deleterious effects (such as with uric acid nephropathy, gouty arthritis, or urolithiasis).

The presence of uric acid in physiologic fluids may be due to several mechanisms, including passive transudation from plasma, active transport of plasma urate (as occurs in the proximal tubule of the kidney), or local production by xanthine oxidase. Active (14) and passive (15) transport mechanisms for plasma and glandular proteins has been observed in human nasal mucosa. Either type of nasal mucosal transport mechanism could exist for uric acid. In addition, local induction of xanthine oxidase activity by several stimuli has been demonstrated in a variety of animal tissues and fluids (30–38), including gastrointestinal mucosa (36) and murine bronchoalveolar lavage fluid (37). Such localized xanthine oxidase activity could occur in the human nasal mucosa, providing another explanation for the presence of uric acid in nasal lavage fluid.

Evidence presented here indicates that the uric acid content of nasal secretions is enhanced by cholinergic stimulation. Nasal submucosal gland secretion is also induced by direct (14–16) and gustatory reflex-associated (17) cholinergic stimulation. These observations suggest that uric acid is a constituent of nasal submucosal gland secretions. Consequently, increases in nasal submucosal gland secretion are the most likely source for cholinergically induced increases of uric acid in nasal lavage fluid. However, the precise mechanism of cholinergic enhancement of nasal mucosal urate secretion remains to be elucidated.

Airways are lined by mucosal surfaces that may be subjected to oxidant stress from ozone (5), oxidants generated by inflammatory cells (6, 7), and iatrogenically administered oxygen (1–3). Significant respiratory diseases that are caused or exacerbated by oxidant exposure include asthma (5), adult respiratory distress syndrome, and BPD (1–3). Tracheal aspirates from infants with BPD have significantly less lactoferrin and lysozyme than those from neonates who do not develop this disorder despite equivalent oxygen exposure

(19). This observation suggests a defect in airway submucosal gland function that predisposes these infants to BPD. As uric acid is probably secreted by airway submucosal glands, it is likely that the absence of uric acid contributes to this predisposition.

The demonstration that uric acid is the only stable, low molecular weight antioxidant in the soluble fraction of nasal secretions suggests that uric acid plays a major role in antioxidant protection of airway surfaces. Additionally, uric acid is an interesting antioxidant for a variety of reasons. First, unlike other antioxidants (such as glutathione), it is metabolically stable. Second, urate is nontoxic while in solution. Additionally, should it crystallize on a mucosal surface, uric acid would be easily removed and, consequently, would not pose the same problems that it does when crystallized in a closed tissue space.

The contributions of both xanthine oxidase and uric acid in oxidative host defense and inflammatory homeostasis in mucous membranes remain to be explored. Studies of antioxidants in airway secretions and mucosa from patients with BPD, asthma, and adult respiratory distress syndrome, as well as normal volunteers, are needed to address these issues. Additionally, the potential pharmacologic use of uric acid as a topical antioxidant presents an interesting possibility but remains uninvestigated.

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