

RESEARCH ARTICLE

Airway purinergic responses in healthy, atopic nonasthmatic, and atopic asthmatic subjects exposed to ozone

Charles R. Esther Jr.¹, David B. Peden², Neil E. Alexis², and Michelle L. Hernandez²

¹Pediatric Pulmonology, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA, and ²Center for Environmental Medicine Asthma and Lung Biology, University of North Carolina School of Medicine, Chapel Hill, NC, USA

Abstract

Context: Ozone exposure triggers airway inflammatory responses that may be influenced by biologically active purine metabolites.

Objective: To examine the relationships between airway purine metabolites and established inflammatory markers of ozone exposure, and to determine if these relationships are altered in individuals with atopy or asthma.

Materials and methods: Mass spectrometry was utilized to measure concentrations of purine metabolites (adenosine monophosphate [AMP], adenosine, hypoxanthine, uric acid) and non-purine metabolites (taurine, urea, phenylalanine, tyrosine) in induced sputum obtained from 31 subjects with normal lung function (13 healthy controls, eight atopic nonasthmatics, and 10 atopic asthmatic [AA]) before and 4 h after ozone exposure.

Results: At baseline, the purines AMP and hypoxanthine correlated with multiple inflammatory markers including neutrophil counts and the cytokines interleukin (IL)-6, IL-8, tumor necrosis factor alpha (TNF- α), and IL-1 β (r ranged from 0.41 to 0.66, all $P < 0.05$). Following ozone exposure, these purines remained correlated with IL-6, IL-8, and TNF- α ($r = 0.37$ – 0.68). However, AMP and hypoxanthine increased significantly post ozone exposure in atopic nonasthmatics but not in AA. In contrast, the non-purine metabolite taurine correlated with baseline neutrophil counts ($r = 0.56$) and IL-6 ($r = 0.53$) and was elevated post-exposure in both atopic cohorts.

Discussion and conclusions: The purine metabolites AMP and hypoxanthine are correlated with multiple inflammatory markers at baseline and after ozone exposure. However, changes in these purine metabolites after ozone appear to differ from other inflammatory markers, with less response in AA relative to atopic nonasthmatics. Purine metabolites may play a role in the signaling responses to ozone, but these responses may be altered in subjects with asthma.

Keywords: Induced sputum, adenosine, adenosine monophosphate, hypoxanthine, taurine

Introduction

Ozone is a common environmental pollutant that has been strongly linked to increased morbidity from respiratory diseases such as asthma and chronic obstructive pulmonary disease (Ciencewicz et al., 2008). Exposure to ozone leads to oxidative stress that can trigger inflammatory airway responses, including neutrophilic inflammation (Hollingsworth et al., 2007). Recent evidence suggests that these inflammatory responses are enhanced in sensitive individuals such as those with atopy or asthma (Hernandez et al., 2010).

Previous studies suggested that exposure to ozone is mediated through production of “danger signals” of oxidative stress that lead to innate immune responses (Depuydt et al., 2002). Interestingly, extracellular purines—biologically active molecules including adenosine triphosphate (ATP) and its metabolites—have also been shown to function as danger signals in response to a variety of noxious stimuli (Bours et al., 2006). More directly, exposure of airway epithelial cells to ozone has been shown to increase release of ATP into the extracellular space (Ahmad et al., 2005). Furthermore, the purine metabolites that result

Address for Correspondence: Charles R. Esther, Pediatric Pulmonology, CB#7217, 5th Floor Bioinformatics, 130 Mason Farm Road, University of North Carolina at Chapel Hill, Chapel Hill, NC 27517, USA. Tel: 919 966-1055. Fax: 919 966-6179. E-mail: Charles_Esther@med.unc.edu

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Abbreviations

AMP, adenosine monophosphate;

ATP, adenosine triphosphate;

IL, interleukin;

TNF, tumor necrosis factor

from metabolism of this extracellular ATP (Burnstock, 2006a) have also been linked to physiological pathways relevant to oxidative stress responses. For example, adenosine monophosphate (AMP) has been shown to correlate with markers of neutrophilic airway inflammation (Esther et al., 2008a; Loughlin et al., 2010), which increase in response to ozone exposure (Seltzer et al., 1986). Adenosine-mediated signaling also influences a variety of inflammatory responses (Mohsenin and Blackburn, 2006), and airway concentrations of this purine are elevated in stable asthmatic subjects (Driver et al., 1993; Huszár et al., 2002; Esther et al., 2009; Loughlin et al., 2010) and are sensitive to exacerbations and effective treatment (Csoma et al., 2005). Adenosine can be further metabolized to hypoxanthine and uric acid, compounds that have also been linked to responses to oxidative stress (Park et al., 2009), with uric acid a known activator of inflammasomes (Gasse et al., 2009).

These findings suggest that purine metabolites may be involved in the response to ozone exposure, and that purinergic responses might be enhanced in sensitive subjects with atopy and/or asthma. To address these hypotheses, we examined sputum supernatants available from a previously published study of ozone exposure in a group of subjects with normal lung function that included healthy normal volunteers (NV), atopic nonasthmatics, and atopic asthmatics (AA) (Hernandez et al., 2010). These samples were analyzed using a mass spectrometric method capable of measuring multiple purine metabolites including AMP, adenosine, hypoxanthine, and uric acid. In addition, measurement of several non-purine metabolites (urea, taurine, phenylalanine, tyrosine) for which mass spectrometric methods have been established were included to assess whether observed relationships were specific to purines. These non-purine metabolites included the sulfa-amino acid taurine that has previously been linked to neutrophilic inflammation (Learn et al., 1990; Witko-Sarsat et al., 1995).

Methods

Subjects and samples

Subjects represented a subset of participants in a previously published study of ozone exposure (13 healthy NV, 8 atopic nonasthmatics with allergic rhinitis [AR], and 10 AA) (Hernandez et al., 2010) in whom induced sputum supernatants obtained before and after ozone exposure were available. As previously described, the groups were defined on the basis of skin prick testing (atopic vs. nonatopic) and methacholine challenge

(asthmatic vs. nonasthmatic). All asthmatics also had a physician diagnosis of mild intermittent asthma and were not on any long-term controller medication (inhaled corticosteroids or leukotriene receptor antagonist). Atopic subjects could utilize antihistamines or topical nasal steroids to control AR. All subjects were free of respiratory symptoms for at least 4 weeks prior to the study. Demographic features of these subjects are shown in Table 1. Each subject was exposed to 0.4 ppm ozone for 2 h, while performing four 15-min sessions of intermittent moderate exercise separated by 15 min of seated rest. Sputum was obtained pre-exposure and again 4–6 h post-exposure. Methods for sputum induction and processing, lung function testing, and assessing sputum cell counts and cytokine concentrations were as previously described (Hernandez et al., 2010); the reference set for percent predicted lung function values was from Knudson (Knudson et al., 1976). Samples were collected between April 2006 and August 2009 and stored at -80°C until analysis in October 2010. There were no significant group differences in time of storage, and no significant correlations were observed between time of storage and concentrations of any metabolite.

Mass spectrometric analysis

Prior to analysis, a solution of isotopically labeled internal standards was added to each sputum supernatant sample to a final concentration of 50 nM [^{13}C , ^{15}N] adenosine, 250 nM [^{15}N] AMP, and 50 μM [^{13}C] urea plus the working concentration of an isotopically labeled amino acid solution (NSK-A; Cambridge Isotope Laboratories, Inc., Andover, MA). Sample plus internal standard was then filtered through a 10-kDa column for 30 min at 11,000 g at 4° to remove macromolecules.

Mass spectrometry was performed using an liquid chromatography-tandem mass spectrometry system with electrospray ionization interface including an Acquity solvent manager and a TSQ-Quantum Ultra Triple Quadrupole Mass Spectrometer (Thermo, San Jose, CA). Five microliters of sample was injected onto an Atlantis T3 C18, 1.8 μm column (Waters, Milford MA) operated with a methanol and 0.1% formic acid gradients as previously described (Esther et al., 2008b). Mass spectrometric detection (Figure 1) was performed in multiple-reaction monitoring mode using appropriate transitions in mass-to-charge ratio (m/z) from parent to production during tandem mass spectrometry for the purine metabolites AMP (m/z transition 348 $\rightarrow$ 136), adenosine (268 $\rightarrow$ 136), hypoxanthine (135 $\rightarrow$ 92), and uric acid (167 $\rightarrow$ 124) plus a select group

Table 1. Demographic characteristics and response to ozone.

Demographics	All subjects	Divided by cohort		
		NV	AR	AA
<i>n</i>	31	13	8	10
Age (years)	24.9 ± 5.6	23.7 ± 5.0	25.3 ± 5.4	26.1 ± 6.8
Gender	16 Males/15 Females	6 Males/7 Females	5M/ 3F	5M/ 5F
Race (% whites)	64.5%	61.5%	62.5%	70.0%
Body mass index (BMI)	26.2 ± 5.4	27.0 ± 6.1	26.2 ± 5.6	25.2 ± 4.6
Response to ozone	All	NV	AR	AA
% Predicted FEV ₁ – pre O ₃	100.1 ± 12.1	102.1 ± 12.7	101.2 ± 12.1	96.8 ± 12
% Predicted FEV ₁ – post O ₃	83.8 ± 13*	84.2 ± 13.3*	87.1 ± 9.0*	80.8 ± 15.6*
PMN counts – pre O ₃	564 ± 736	470 ± 660	560 ± 780	680 ± 860
PMN counts – post O ₃	928 ± 992*	720 ± 710	920 ± 1140	1160 ± 1170

NV = healthy normal volunteers, AR = atopic nonasthmatics (allergic rhinitis), AA = atopic asthmatics, O₃ = ozone, PMN = neutrophil (polymorphonuclear leukocyte).

**P* < 0.05 versus pre O₃.

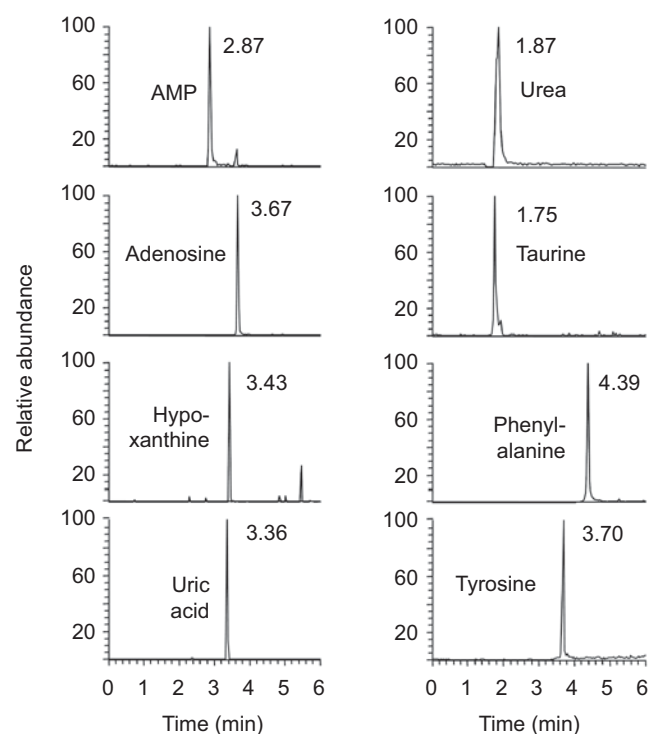


Figure 1. Mass spectrometric chromatogram demonstrating detection of several purine metabolites (left) and non-purine metabolites (right) from a sputum sample. The column run time for each peak is shown.

of non-purine metabolites with which we have previous experience including urea (61→44), taurine (124→80), phenylalanine (166→120), and tyrosine (182→136). In addition, ion transitions were monitored for the isotopically labeled AMP (353→141), adenosine (283→151), urea (62→45), phenylalanine (172→126), and tyrosine (188→171) as internal standards. All ions were monitored in positive mode except for hypoxanthine, uric acid, and taurine, which were monitored in negative mode. Mass spectrometric signal was defined as the ratio

of the peak area of the metabolite divided by the peak area of the relevant internal standard. Hypoxanthine and uric acid utilized the adenosine internal standard and taurine the urea internal standard based on similar column run times. Metabolite concentrations were determined by analysis of standard solutions run in parallel.

All compounds were detected in all samples (Figure 1), except for one sample in which taurine was below detection limits. In addition, 10 of the 62 samples analyzed (three healthy control, two atopic nonasthmatic, five AA samples) exhibited extensive degradation of the AMP internal standard (but not adenosine or other internal standards), suggesting high ectonuclease activity. As this high activity could alter concentrations of both AMP and adenosine (the product of AMP hydrolysis), these samples were excluded from analysis for adenosine and AMP.

Statistical analysis

Data are presented as mean ± standard deviation. All sputa data were log-transformed prior to analysis to yield normalized datasets, as determined by the D'Agostino and Pearson omnibus normality test. Correlations were determined using Pearson correlation coefficients. Changes after ozone exposure were assessed using paired Student's *t*-tests.

Results

Sputum supernatant samples obtained before and after ozone exposure were available from 31 subjects including 13 healthy controls, eight atopic nonasthmatics, and 10 AA. These subjects represented a subset of the subjects included in a previously published study (Hernandez et al., 2010), and demographic characteristics in this subset were similar to those of the larger group and did not exhibit any significant between group differences (Table 1). Lung function as assessed

by percent predicted FEV₁ was within the normal range at baseline but significantly decreased post ozone exposure in the group as a whole and in all individual cohorts (Table 1). Sputum neutrophil counts (cells/mg) also increased after ozone exposure when all subjects were combined ($P < 0.01$), although increases observed within the individual cohorts did not reach statistical significance. Of note, increases in neutrophil counts post ozone exposure reached statistical significance in atopic nonasthmatic and AA cohorts within the larger study (Hernandez et al., 2010).

Because several purine metabolites have been previously linked to inflammatory responses (Burnstock, 2006b), the relationships between sputum purine metabolites and established markers of inflammation were assessed. In the baseline, pre-exposure samples, the purine metabolites AMP and hypoxanthine were significantly correlated with multiple markers of inflammation including sputum neutrophil counts and the cytokines interleukin (IL)-1 β , IL-6, IL-8, and tumor necrosis factor alpha (TNF- α) (Table 2). In addition, adenosine was statistically significantly correlated with the cytokines IL-6 and IL-8, with a correlation to neutrophil counts that did not reach statistical significance ($r = 0.40$, $P = 0.0504$). In contrast, no significant correlations were observed between uric acid and any inflammatory marker (data not shown). Among the non-purine metabolites, only taurine exhibited any significant correlation to inflammatory markers with correlations to neutrophil counts and IL-6 (Table 2). Phenylalanine, tyrosine, and urea were not correlated with any inflammatory marker (data not shown).

In the sputum samples obtained post ozone exposure, the purine metabolites AMP and hypoxanthine remained significantly correlated with the cytokines IL-6, IL-8, and TNF- α (Table 2). Adenosine was not correlated with any inflammatory marker post ozone exposure. Of the non-purine metabolites, taurine remained correlated with IL-6 and exhibited a modest correlation with TNF- α . No purine or non-purine metabolite was correlated with sputum neutrophil counts post ozone exposure, and the metabolites uric acid, phenylalanine, tyrosine, and urea were not

significantly correlated with any inflammatory marker in these samples.

These data suggested that sputum concentrations of AMP and hypoxanthine, and to a lesser extent adenosine and taurine, were significantly correlated with markers of inflammation both before and after ozone exposure. Therefore, we examined whether exposure to ozone altered concentrations of any of these metabolites comparable with the statistically significant increases we observed in neutrophil counts (Table 1) and in the cytokines IL-6 (138 ± 141 pg/mL pre, 264 ± 360 pg/mL post, $P < 0.05$) and IL-8 (24.2 ± 32.4 ng/mL pre, 35.9 ± 46.4 ng/mL post, $P < 0.05$) within the group as a whole. Interestingly, although we observed the predicted increase in sputum taurine following ozone exposure (Table 2), changes in the purines AMP and hypoxanthine did not reach statistical significance. Because our previous study suggested that inflammatory responses to ozone are increased in subjects with atopy and/or asthma (Hernandez et al., 2010), we re-examined the data with the groups stratified by cohort. In these evaluations, significant increases in AMP and hypoxanthine were observed in the atopic nonasthmatics, but not in healthy controls or AA (Figure 2A–2C). Overall, the magnitude of change in hypoxanthine was higher in atopic nonasthmatics (increase of 0.24 ± 0.22 log units) than in AA (-0.23 ± 0.48 log units, $P < 0.05$), with similar pattern for AMP (increase of 0.34 ± 0.31 log units atopic nonasthmatics, 0.00 ± 0.29 log units AA) not reaching statistical significance ($P = 0.095$). In contrast, taurine increased significantly in both atopic nonasthmatic and AA cohorts (Figure 2D), and the magnitude of change was similar in both atopic cohorts (increase of 0.32 ± 0.34 log units in AA, 0.23 ± 0.28 log units in atopic nonasthmatics, $P = 0.60$). No statistically significant changes for any purine or non-purine compound were observed in healthy normal volunteers, and no statistically significant changes in adenosine (Figure 2B), uric acid, urea, phenylalanine, or tyrosine (data not shown) were observed in any cohort. Of note, we did not observe any statistically significant differences in baseline pre-exposure metabolite concentrations, with an approximately ~ 0.2 log unit increase in the asthma cohort relative to the healthy controls not reaching statistical significance ($P = 0.25$).

Table 2. Purine metabolite correlations to inflammatory markers and concentrations pre and post ozone exposure in all subjects.

	AMP		Adenosine		Hypoxanthine		Taurine	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post
Correlations pre and post ozone exposure								
PMNs/mg sputum	0.41	n.s.	n.s.	n.s.	0.66	0.38	0.56	n.s.
IL-1 β	0.41	n.s.	n.s.	n.s.	0.56	n.s.	n.s.	n.s.
IL-6	0.62	0.52	0.46	n.s.	0.66	0.68	0.63	0.53
IL-8	0.41	0.47	0.43	n.s.	0.66	0.37	n.s.	n.s.
TNF- α	0.53	0.63	n.s.	n.s.	n.s.	0.48	n.s.	0.52
Concentrations pre and post ozone exposure								
Concentration (nM)	195 $\pm$ 169	308 $\pm$ 343	348 $\pm$ 312	321 $\pm$ 378	286 $\pm$ 243	369 $\pm$ 360	609 $\pm$ 499	897 $\pm$ 665*

AMP, Adenosine monophosphate; IL, interleukin; TNF- α , tumor necrosis factor alpha; PMN, polymorphonuclear leukocytes. For correlations, $P < 0.05$ for all numeric values, bold values $P < 0.01$. For concentrations, all values are shown. * $P < 0.01$ versus Pre.

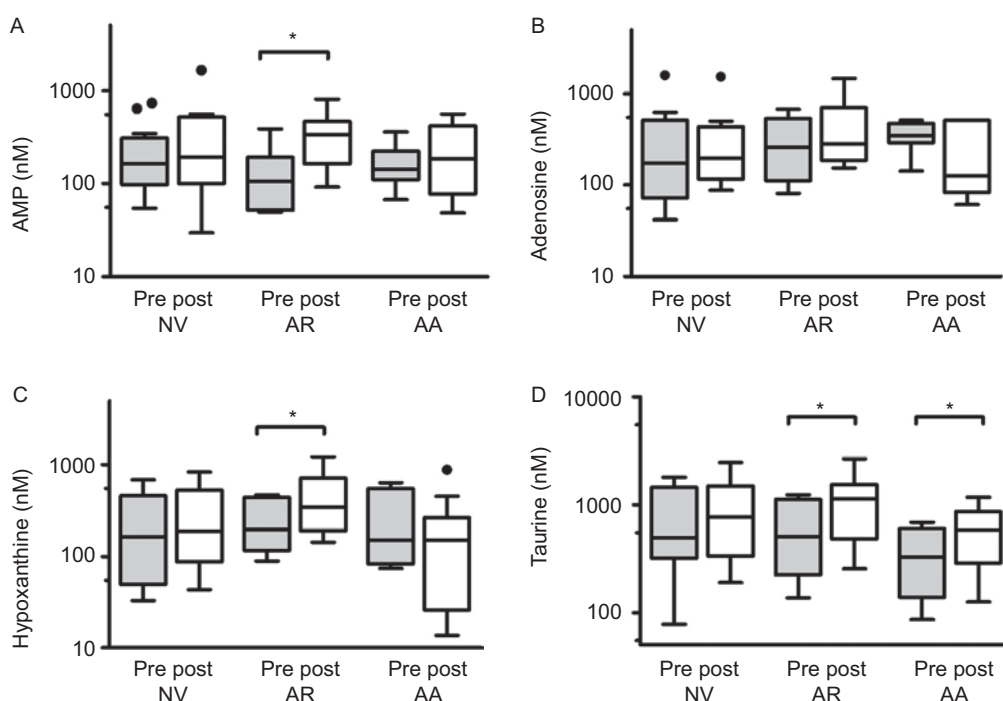


Figure 2. Concentrations pre (gray bars) and post (white bars) ozone exposure were analyzed for the metabolites correlated with neutrophil counts in baseline samples: AMP (A), Ado (B), hypoxanthine (C), and taurine (D). Significant increases after ozone exposure were noted for AMP and hypoxanthine in the atopic nonasthmatics with allergic rhinitis (AR) but not in the atopic asthmatics (AA) or healthy normal volunteers (NV). In contrast, sputum taurine increased significantly in both atopic groups. Data shown are a box plot with whiskers as defined by Tukey (1.5× the intraquartile range). * $P < 0.05$ pre versus post, with analyses performed on log-transformed mean values not depicted in the figure.

Discussion

In this study, we observed that two purine metabolites—AMP and hypoxanthine—were significantly correlated with multiple inflammatory markers that included neutrophil counts and proinflammatory cytokines both at baseline and following exposure to inhaled ozone. These correlations were particularly robust for the cytokines IL-6 and IL-8, which have been well-established as biomarkers of ozone-induced inflammation (Devlin et al., 1991; Jaspers et al., 1997; Hernandez et al., 2010). Interestingly, although adenosine has also been linked to ozone-mediated inflammatory responses (particularly via IL-6) (Sitaraman et al., 2001; Sun et al., 2008), the correlations between adenosine and inflammatory markers were less robust than for other purine metabolites in this study. Similarly, no relationships were observed between uric acid and inflammatory markers despite previous studies suggesting that uric acid concentrations may influence inflammasome function (Gasse et al., 2009). However, the samples obtained during this study reflected only a single, relatively early (4 h) time point after ozone exposure, and it is possible that some relationships between purine metabolites and ozone-mediated inflammatory processes may be present at other time frames. Furthermore, since subjects exercised during ozone exposure, we cannot rule out the possibility that exercise contributed to the observed changes. However, we note that exercise

alone did not induce changes in sputum inflammatory markers in a previous similar study (Lay et al., 2007).

The correlations we observed between inflammatory markers and the purine metabolites AMP and hypoxanthine are consistent with findings from previous studies. Correlations between AMP and neutrophils have been observed in several studies of respiratory samples (Eltzschig et al., 2006; Esther et al., 2008a; Loughlin et al., 2010), and elevated AMP concentrations have been detected in airway samples from patients with cystic fibrosis (CF), a disease associated with significant neutrophilic airway inflammation (Esther et al., 2008a, 2009). The mechanisms that underlie these relationships are not clear, but may reflect release of ATP and accumulation of AMP directly by neutrophils (Lennon et al., 1998; Chen et al., 2006). Indeed, ozone has been shown to trigger release of ATP from airway epithelial cells (Ahmad et al., 2005), suggesting that the purine metabolites measured in this study may be derived from metabolism of ATP. Unfortunately, the mass spectrometric method used in this study was not sufficiently sensitive to measure ATP directly. The relationships between hypoxanthine and airway inflammation have been less well-defined, but this purine metabolite is strongly linked to oxidative injury (Park et al., 2009) and has been found to be elevated in plasma and bronchoalveolar lavage fluid from patients with acute respiratory distress syndrome (Quinlan et al., 1997).

Interestingly, as AMP and hypoxanthine were correlated with multiple inflammatory markers before and after ozone exposure, statistically significant increases in these metabolites after exposure to ozone were not observed within the group as a whole. Examination of the group stratified by cohort suggested that the responses of hypoxanthine (and likely AMP) were diminished in the AA cohorts relative to the atopic nonasthmatics. This contrasts to our experience with other inflammatory markers, in which the responses to ozone are generally enhanced in AA (Hernandez et al., 2010).

These findings suggest the possibility that the purinergic component of the response to ozone may be reduced in the airways of subjects with atopic asthma relative to other atopic individuals. If true, a reduced purinergic response to ozone in asthmatics would be somewhat surprising, since several studies have demonstrated that airway adenosine concentrations are elevated in asthmatic subjects (Driver et al., 1993; Huszár et al., 2002; Loughlin et al., 2010) and increase after exposure to bronchoconstrictive stimuli such as exercise or allergen (Huszár et al., 2002; Vizi et al., 2002; Csoma et al., 2005). However, responses to ozone differ from those to other stimuli, with a more prominent neutrophilic component in contrast to the predominant eosinophilic response to allergen (Ciencewicz et al., 2008). Indeed, as adenosine has been identified as the primary purine altered in asthma and other diseases, we did not observe changes in adenosine after ozone exposure in the group as a whole or within any cohort, although this relatively small exploratory study may have been insufficiently powered to detect these changes.

Purinergic signaling pathways are known to modulate inflammatory responses (Burnstock, 2006b), suggesting that the purinergic responses observed in this study may influence other inflammatory cascades. In particular, adenosine-mediated signaling has been shown to have both positive and negative immunomodulatory effects (Vass and Horváth, 2008), which can be mediated both by adenosine present in extracellular fluid and by local generation of adenosine from AMP via nuclease activity at epithelial surfaces (Eltzschig et al., 2006). As adenosine-mediated signaling alone tends to increase production of proinflammatory cytokines such as IL-6 (Sun et al., 2008), increased concentrations of adenosine have also been shown to attenuate release of IL-6 in response to other inflammatory stimuli such as lipopolysaccharide (LPS) (Haskó et al., 1996). Consistent with these observations, the AA group that appeared to have decreased purinergic responses in this study also had the largest increase in ozone-stimulated IL-6 production in the larger study from which these samples were drawn (Hernandez et al., 2010). As these associations between purines and other inflammatory mediators are intriguing, more definitive elucidation of these relationships will require further study.

It is feasible that the findings of this study are non-specific artifacts of the sample collection or analytic methods. However, the fact that we did not observe correlations between inflammatory markers and several non-purine metabolites (urea, phenylalanine, tyrosine) argues against this hypothesis. Furthermore, the relationships between sputum taurine and markers of inflammation are consistent with previous studies of this metabolite. Taurine is found at high concentrations within human neutrophils (Fukuda et al., 1982), and elevated sputum taurine concentrations have been observed to correlate with markers of inflammation in CF patients (Witko-Sarsat et al., 1995). Thus, the relationships between sputum taurine and neutrophil counts and its increase after ozone exposure are consistent with predictions and lend further credence to the analytic methods used in this study.

Conclusions

In summary, our study suggests that purine metabolites—particularly AMP and hypoxanthine—are correlated with inflammatory markers of ozone exposure. However, the analyses suggest the possibility that the purinergic component of the response to ozone may be diminished in AA relative to other atopic individuals. Being interesting, these findings will need to be confirmed through further study in a larger cohort of individuals.

Declarations of interest

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