

ORIGINAL ARTICLE

Increased Plasma Reactive Oxidant Levels and Their Relationship to Blood Cells, Total IgE, and Allergen-specific IgE Levels in Asthmatic Children

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Asthma is a disorder characterized by inflammation of the airways. Oxidative stress may play a role in the pathophysiology of several diseases including asthma. Characterizing biomarkers of oxidative stress in the context of other systemic measures of immune function or inflammation could provide insight regarding underlying mechanisms inducing asthma. We evaluated whether oxidative stress in the form of plasma reactive oxidants differs between asthmatic and non-asthmatic children and elucidate relationships between plasma reactive oxidants and other asthma-related immunological markers. Plasma reactive oxidants, white blood cell counts, total serum immunoglobulin E (IgE), and a multi-allergen-specific IgE screen were measured in 74 asthmatic and 74 non-asthmatic children (9 to 13 years of age) from the Detroit, Michigan area. Plasma reactive oxidants were measured using a lucigenin-based chemiluminescence assay. Plasma reactive oxidants, eosinophils, and neutrophils (absolute counts and percent of total white blood cell counts), total IgE, and allergen-specific IgE levels were elevated in asthmatics after adjusting for age, gender, and ethnicity. IgE (total or allergen-specific), eosinophils and neutrophils were not significantly associated with plasma reactive oxidant levels. The association between plasma reactive oxidants and asthma status was similar when eosinophils, neutrophils, total IgE, or allergen-specific IgE were included as possible confounders in multivariate logistic regression models. In conclusion, plasma reactive oxidants are elevated in asthmatics and appear to be an independent predictor of asthma status. Measurement of plasma reactive oxidants may be a useful adjunct diagnostic tool and potential mechanistic indicator relevant to the study of asthma and asthma exacerbation.

Keywords asthma; reactive oxidants; oxidative stress; immunoglobulin E; inflammation

INTRODUCTION

Asthma is a disease involving chronic airway inflammation and dysfunction.

Inflammation in a majority of asthma cases is IgE antibody mediated and is thought to be perpetuated by the presence of elevated numbers of eosinophils (1), a hallmark of asthma status. Non-eosinophilic asthma has been proposed as a distinct asthma phenotype (2) and is often characterized by neutrophilic inflammation, which can act in cooperation with environmental factors and eosinophilic inflammation in severe asthmatics (2, 3). Activated inflammatory cells, including neutrophils and eosinophils, generate reactive oxidants (RO) that produce oxidative stress and can elicit a number of gene transcription and cell signaling changes (4). RO are thought to mediate inflammation

through cell signaling in several disease processes throughout the body, including cancer (5). Granulocytic cells, and eosinophils in particular, spontaneously generate superoxide, a type of RO, at sites of antigen challenge, which contributes to inflammation-induced airway injury (6). Certain asthma medications have been shown to reduce superoxide production, which may contribute to their anti-inflammatory properties (7–9). Synthetic antioxidant mimetics that block RO-related damage are also being considered for asthma treatment (10). Asthmatics display increased generation of superoxide in inflammatory cells in both the lung and blood compartments (11–14), that correlates with measures of disease severity (15–17). Increased levels of RO from peripheral blood cells are present in both stable and exacerbated asthmatics relative to healthy control subjects (13–16). Recently Suzuki et al. (18) reported elevated levels of reactive oxygen metabolites in serum in subjects exhibiting acute exacerbation of asthma. These studies suggest that RO measurement may be used to distinguish asthma status.

Chemiluminescent measurement of RO is an effective and relatively simple procedure. Chemiluminescence (CL)-based RO measurements are generally evaluated in cell types relevant to the disease being studied, e.g., immune cells from patients with Crohn's disease (19), adult respiratory distress syndrome (19), or acute myeloid leukemia (20). Several

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studies have used lucigenin-based CL to detect superoxide production in peripheral blood cells collected from asthmatics (13, 14, 22) rather than using more invasive lung lavage techniques. Because diagnostic indicators such as cholesterol and triglycerides are measured in serum (23), it is of interest to ascertain whether RO is measurable in the same specimens. Plasma RO may reflect a more integrated measure of oxidative stress across multiple organ systems (24–26).

Eosinophils and neutrophils are associated with asthmatic inflammation. Higher blood eosinophil levels are associated with increased asthma severity (27), whereas non-eosinophilic asthmatics can exhibit elevated neutrophil levels (2). Allergic disease has been strongly associated with asthma. Allergen cross-linking of IgE antibody on effector cells induces allergic inflammation, one of the strongest susceptibility factors for the development of asthma (26). This study examines cell and serum measures of allergic inflammation in the context of plasma RO levels in asthmatic and non-asthmatic children. Plasma RO levels were evaluated in relation to other indicators of asthma symptomatology (total serum IgE, a panel of allergen-specific serum IgEs, eosinophils, and neutrophils) to elucidate whether these markers are independent predictors of asthma.

METHODS

Study Population and Recruitment

The Environmental Protection Agency (EPA) conducted the Mechanistic Indicators of Childhood Asthma (MICA) in Detroit, Michigan: a subset of children who previously participated in the Detroit Children's Health Study (DCHS). For DCHS recruitment, questionnaires were mailed to members of the Henry Ford Health System (HFHS), Detroit, MI). MICA participants were recruited from households that returned DCHS questionnaires and had clinical visits between November 2006 and January 2007. MICA participants were prioritized to ensure equal participation of asthmatics and non-asthmatics.

Asthma status was based on both clinical records and a parental questionnaire. A child was considered asthmatic if the clinical record showed one or more asthma-related emergency department visits, two or more asthma-related outpatient visits, or two or more asthma-related medications. From the parental questionnaire, a child was considered asthmatic with a parental report of a physician's diagnosis of asthma. Children with conflicting or incomplete asthma status data were excluded from the analyses. The MICA study data include results from 148 asthmatic and non-asthmatic children 9 to 13 years of age residing in Detroit and Dearborn, Michigan. We classified asthmatic children who never used asthma medication ($N = 6$) or occasionally ($N = 27$) as mild asthmatics, while those who took one or more asthma medications on a daily basis ($N = 41$) were described as moderate to severe asthmatics. The study design and protocols were approved by the Institutional Review Boards at HFHS (Detroit, MI), Westat Inc. (Rockville, MD), and the University of North Carolina at Chapel Hill (Chapel Hill, NC—EPA's IRB of record). Written consent was obtained from guardians and child with an oral review of both consent and assent before drawing blood.

Immune Function Assessment

Whole blood (2 mL) was collected into EDTA-containing Vacutainer tubes (BD, Franklin Lakes, NJ) and a complete blood count with differentials was performed by the Henry Ford Health System clinical laboratory.

Serum specimens were prepared by collecting whole blood (8 mL) in a Vacutainer serum clot, red/gray tube (BD, Franklin Lakes, NJ) for each child. Serum was separated following centrifugation, and a 1.7 mL aliquot of serum was frozen at -80°C and sent to Johns Hopkins University School of Medicine Dermatology, Allergy and Clinical Immunology (DACI) Reference Laboratory. Total IgE and a multi-allergen screen for allergen-specific IgE antibody specific for a panel of aeroallergens were quantified in the ImmunoCAP 250 autoanalyzer (Phadia, Portage, Michigan). The multi-allergen screen detects the presence (positive/negative) and measures the relative level of IgE antibody specific for a panel of at least 15 common aeroallergens (Phadiatop). Each subject was tested with the same panel of aeroallergens. Phadiatop levels greater than 0.35 kIU/L indicate the presence of IgE antibody and provide a general assessment of the subject's atopic status. Phadiatop levels exceeding 100 kIU/L were set to 100 kIU/L. Daily quality control was performed with Levey-Jennings plots for human sera that contained known amounts of IgE antibody.

Chemiluminescence Assay for Plasma Reactive Oxidants

Whole blood (2.5 mL) was collected in foil-wrapped Vacutainer tubes containing sodium citrate and was put on ice and plasma immediately separated from the whole blood. After centrifugation for 10 minutes at $2500 \times g$, a 250- μL aliquot of plasma was removed and protected from light exposure. The maximum time allowed between blood draw and the initiation of storage at -80°C was 60 minutes, but the time elapsed was 15 to 30 minutes for most samples. Plasma was stored for 72 to 79 days at -80°C before analysis.

Lucigenin reportedly reacts primarily with superoxide in human plasma (28, 29), however, the oxidant's status may have been altered as a result of freezing. Therefore, in this report, we refer to the substances reacting with lucigenin in this assay as "reactive oxidants" or RO. The chemiluminescence measurement of RO in plasma was adapted from Wu et al. (24), with minor modifications. Aliquots of 64 μL (in triplicate) of either plasma or $1 \times$ phosphate buffered saline (PBS) were added to a 96-well plate, mixed with 160- μL of freshly prepared lucigenin (2 mg/mL in $1 \times$ [PBS]; Sigma, St. Louis, MO). Chemiluminescence measurements were performed at 37°C using an LB96V MicroLumatPlus luminometer (Berthold Technologies). One minute after lucigenin addition, chemiluminescence was measured at 10-second intervals for a total of 10 minutes. Light emission units (relative luminescence units, RLU) were summed and averaged over the 10 minutes of measurement. Measurements from the PBS control subjects were subtracted from plasma RO measurements to adjust for background luminescence. Measurements less than background controls ($N = 2$) were set to equal the lowest detected value in the data set (0.6 RLU).

Statistical Analyses and Variables

Statistical analyses were performed using SAS 9.1 (SAS Institute, Cary, NC). Total serum IgE and allergen-specific IgE (Phadiatop) were modeled as their natural logarithms, whereas eosinophils and neutrophils variables were modeled as the percentage of absolute eosinophils or absolute neutrophils in relation to total white blood cell count. Differences in means were assessed using Student's *t* tests, applying the Satterthwaite method for groups with unequal variance and the pooled method for groups with equal or similar variance between the groups. Associations between biological indicators (RO, eosinophils, neutrophils, total serum IgE, and allergen-specific IgE) and asthma were examined using multivariate logistic regression analysis. We also examined whether the relationship between RO and asthma was independent of other clinical markers of asthma (eosinophils, neutrophils, total serum IgE, and allergen-specific IgE) using multivariate logistic regression models that included RO and other clinical markers as potential predictors of asthma status. All multivariate models included fixed covariate terms adjusting for age, sex, and ethnicity. Age was modeled as the natural logarithm of age in months. Odds ratios for all biological indicators were scaled to their respective interquartile ranges (IQR) to facilitate comparisons of the relationships between different biological indicators and asthma. All analyses were considered statistically significant at *p* value ≤ 0.05 .

RESULTS

A total of 148 children with concordant clinical and questionnaire reports of asthma status: 74 with a history of asthma and 74 without a history of asthma were used for this analysis. The cohort was predominantly African-American (88%) and had slightly more boys (59%) than girls.

Univariate distributions and *t* tests for the blood biological indicators by asthma status are shown in Table 1. Mean levels of RO, eosinophils, total serum IgE, and allergen-specific IgE were significantly higher among asthmatics compared

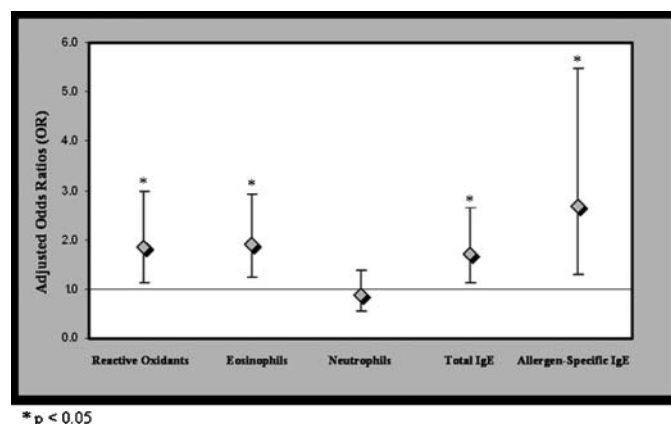


FIGURE 1.—Multivariate logistic regression analysis of blood biological indicators and asthma status; 148 children residing in the Detroit Metropolitan Area. Shown are adjusted odds ratios for multivariate regression analysis of blood indicators and asthma status. The error bars denote 95% confidence intervals. Associations between biological indicators and asthma were statistically significant where the 95% confidence intervals did not cross the horizontal axis at 1.0. RO, eosinophils, total allergen-specific IgE were positively associated with asthma (*p* < 0.01).

with non-asthmatics. There was no significant difference in neutrophil levels between asthmatics and non-asthmatics. Duration of blood plasma storage was not associated with plasma RO concentration (linear regression analysis coefficient: -0.0018 , 95%CI: $[-0.096, 0.092]$). Using the frequency with which subjects took asthma medications as a surrogate measure of asthma severity, RO levels were 6.6 ± 3.1 for mild asthmatics versus 8.8 ± 4.5 for moderate to severe asthmatics (*p* < 0.017).

Associations between biological indicators and asthma are shown in Figure 1. RO was positively associated with asthma adjusting for age, sex, and ethnicity (odds ratio = 1.84; *p* = 0.0069). Eosinophils (odds ratio = 1.90; *p* = 0.0035), total

TABLE 1.—Descriptive statistics for blood indicators by asthma status; 148 children residing in the Detroit Metropolitan Area

	N	Mean (Standard Deviation)	Range	Interquartile Range	P-value for T-Test
Plasma reactive oxidants (RO)*					
All	148	7.1 (3.9)	0.6–22.8	5.1	
Non-Asthmatic	74	6.3 (3.6)	0.6–16.5		
Asthmatic	74	7.8 (4.0)	0.6–22.8		0.017
Total Serum IgE (kUa/L**) [†]					
All	147	4.6 (1.6)	0.7–8.5	2.0	
Non-Asthmatic	74	4.3 (1.6)	0.7–8.5		
Asthmatic	73	5.0 (1.6)	0.7–7.7		0.011
Allergen-specific IgE (kUa/L**) [†]					
All	147	1.4 (2.2)	–1.0–4.6	4.6	
Non-Asthmatic	74	0.9 (2.1)	–1.0–4.6		
Asthmatic	73	1.9 (2.2)	–1.0–4.6		0.0059
% Eosinophils					
All	148	3.6 (2.9)	0–13.0	3.2	
Non-Asthmatic	74	2.8 (2.3)	0–9.4		
Asthmatic	74	4.3 (3.2)	0–13.0		0.002
% Neutrophils					
All	148	49.0 (11.6)	25.5–95.5	15.9	
Non-Asthmatic	74	49.6 (12.4)	25.5–95.5		
Asthmatic	74	48.4 (10.7)	25.9–76.1		0.53

*Relative luminescence units.

**1 U is equivalent to 2.44 ng of IgE.

[†]Natural log transformed.

TABLE 2.—Multivariate logistic regression analysis of RO and eosinophils, neutrophils, total and allergen specific IgE

	RO and Eosinophils ^a	RO and Neutrophils ^b	RO and Total Serum IgE ^c	RO and Allergen-specific IgE ^d
RO	Odds Ratio [95% CI] 1.85* [1.12, 3.04]	Odds Ratio [95% CI] 1.83* [1.13, 2.97]	Odds Ratio [95% CI] 1.86* [1.14, 3.05]	Odds Ratio [95% CI] 1.91* [1.16, 3.14]
% Eosinophils	1.91† [1.23, 2.97]	—	—	—
% Neutrophils	—	0.95 [0.60, 1.50]	—	—
Total serum IgE	—	—	1.73* [1.10, 2.71]	—
Allergen-specific IgE	—	—	—	2.76† [1.32, 5.74]

The association between plasma reactive oxidants and asthma status did not vary when eosinophils, neutrophils, total IgE, or allergen-specific IgE were include as possible confounders in multivariate logistic regression models.

^aModels include RO and eosinophils.

^bModels include RO and neutrophils.

^cModels include RO and total serum IgE.

^dModels include RO and allergen-specific IgE.

* $p < 0.05$.

† $p < 0.10$.

serum IgE (odds ratio = 1.70; $p = 0.016$), and allergen-specific IgE (odds ratio = 2.65; $p = 0.0076$) were also positively associated with asthma in multivariate logistic regression models after adjusting for age, sex, and ethnicity. There was also a slight inverse association between neutrophils and asthma (odds ratio = 0.87); however, this association did not achieve statistical significance ($p = 0.56$).

The association between plasma reactive oxidants and asthma status did not vary when eosinophils, neutrophils, total IgE, or allergen-specific IgE were included as possible confounders in multivariate logistic regression models (Table 2).

DISCUSSION

Asthma is a complex disease characterized by inflammation of the airways. Many cells and cellular constituents are thought to modulate the formation of RO, which in turn may mediate cellular signaling pathways related to immunity and inflammation (5). Characterizing new markers of inflammation in the context of well-established indicators of immune function may improve the diagnostic relevance and mechanistic significance of RO. Such studies may provide insight into the complex interactions between cells and immune and antioxidant factors transported in the plasma that can lead to increased levels of inflammation, a hallmark of many diseases including asthma.

RO levels are elevated in asthmatics and are believed to be involved in the pathogenesis of asthma. For example, resting blood cells from unstable asthmatics exhibit increased levels of oxidants compared with stable asthmatics, who in turn have higher oxidant levels than normal subjects (13). To our knowledge, the present study is the first to examine plasma levels of RO in asthmatic children, in relation to more established clinical indicators of immune function. While whole blood RO has been shown to be elevated in certain disease states (19, 30), including asthma (14), we hypothesized that plasma RO would be elevated in asthmatics owing to the chronic inflammation associated with this disease. A modified chemiluminescence assay (24) was used to measure plasma RO levels. The relationships between plasma RO, total and allergen specific IgE, and inflammatory cells (neutrophils and eosinophils) were evaluated in children 9 to 13 years of age. Elevated levels of plasma RO, total and allergen specific IgE, and eosinophils were observed in asthmatic ver-

sus non-asthmatic children and were statistically significant by t test and multivariate analysis models after adjusting for age, sex, and ethnicity. Total and allergen specific IgE levels, eosinophil and neutrophil levels were not significantly associated with plasma RO levels in this study population (data not shown). Relationships between RO and asthma were similar in statistical models adjusting for eosinophil, neutrophil, total IgE, or allergen-specific IgE levels. These results suggest that plasma RO predicts asthma independently from these serological markers. Our results are consistent with studies by Huang et al. (26) in which plasma RO was compared to two markers of inflammation, C-reactive protein and ferritin. Despite elevated levels of all three markers in febrile emergency room patients, no relationship between RO and these parameters was observed. The regulation of the immune system is complex, with molecular and cellular pathways impacted and modulated by cells and cellular constituents (31).

Consistent with the published literature (1) eosinophil levels were elevated in the asthmatic group. Eosinophils are involved in the inflammatory process and can produce free radicals when activated (32), to a much higher degree than neutrophils (33). This study could not evaluate the extent to which neutrophils and eosinophils act in concert to induce the formation/release of antioxidants or RO.

Total and allergen-specific IgE levels were elevated in asthmatics. Allergen-sensitized individuals are thought to be more susceptible to RO-induced inflammation. This susceptibility appears to be linked to polymorphisms in glutathione-S-transferase that reduce the protein's ability to protect against RO (34). No significant relationship between plasma RO levels and total or allergen-specific IgE levels was observed in the present study. Given that over 95% of the asthmatic subjects in the clinical study were stable, the present study could not evaluate asthma severity or symptoms in terms of their levels of RO or immune function indicators.

Neutrophils are elevated in severe asthmatics, during severe exacerbations, and in asthmatic smokers (27). Asthma characterized by high levels of neutrophils typically manifests among middle-age, non-atopic, female adults (2). Taken together, most children in this study would not have a neutrophilic form of asthma. In the present study, no association was observed between neutrophil and plasma RO levels, consistent with a study by Huang et al. (26) of febrile patients but inconsistent with the findings of Mattheyse et al. (35), where

RO released from neutrophils diminished during episodes of asthmatic airway obstruction. The authors suggested that the activated cells may preferentially sequester in the lung.

The current study has several limitations. Given the single time point blood measurement, intra-individual variability, an important criterion when validating a new biologic or clinical indicator of exposure or a disease state, could not be assessed. Plasma RO levels were not evaluated in relation to other potential indicators of inflammation, e.g., C-reactive protein, ferritin, or cytokines, nor were RO levels studied in relation to lung function. We are intrigued by the detection of RO in frozen plasma, suggesting that the specific RO measured in this assay are stable and therefore not like the commonly repudiated superoxide anion. The detection of RO in frozen plasma by lucigenin-induced chemiluminescence analysis has been reported by Park et al. (25). There is debate as to whether the lucigenin probe overestimates the superoxide levels due to possible redox cycling of lucigenin itself. Ex vivo generation of superoxide can be potentiated via interactions with various cofactors and/or substrates in the plasma (28, 29). Thus, the possibility exists that we are measuring plasma RO and/or RO generation by plasma constituents (cofactors and/or substrates) that predispose to RO formation in the presence of our chemiluminescence agent. The convenience of storing plasma before RO analysis extends the application towards larger clinical or epidemiological studies. Using the frequency with which subjects took asthma medication as a surrogate indicator of asthma severity, RO measurements were significantly higher in moderate to severe asthmatics compared to mild asthmatics. However, the classification of asthma severity based on asthma medication usage alone is limited because we did not control for variation in asthma medication dose, type, and route of administration.

In conclusion, few clinical and environmentally relevant indicators of systemic inflammation have been developed despite the existence of chemicals known to induce reactive oxidants (36) and the association of inflammation with many disease states. Our data indicate that a simple measure of systemic reactive oxidant burden may be clinically useful in assessing asthmatic children. Plasma RO levels were independent of eosinophil and neutrophil concentrations, total IgE, or the presence of allergen-specific IgE antibody; and the association between RO and asthma appeared to be independent of these clinical indicators of asthma. Measuring RO in plasma obviates the need for ex vivo challenge of isolated blood cells with environmental or pro-inflammatory stimuli and may provide a useful adjunct to other serum markers of vascular inflammation. For example, eosinophil cationic protein, a potential biomarker of airway inflammation, is elevated in the serum, plasma, induced sputum, saliva, and bronchoalveolar lavage fluid of stable asthmatics and used to assess asthma severity and management (37). Whether measurement of RO will be useful from a diagnostic or asthma management perspective will require further evaluation. We are presently evaluating RO in other biological fluids to evaluate whether plasma RO is a sensitive marker of exposure to environmentally relevant contaminants in water and air.

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DECLARATION OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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