



Original Contribution

Oxidant stress modulates murine allergic airway responses

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Abstract

The allergic inflammation occurring in asthma is believed to be accompanied by the production of free radicals. To investigate the role of free radicals and the cells affected we turned to a murine model of allergic inflammation produced by sensitization to ovalbumin with subsequent aerosol challenge. We examined oxidant stress by measuring and localizing the sensitive and specific marker of lipid peroxidation, the F₂-isoprostanes. F₂-isoprostanes in whole lung increased from 0.30 ± 0.08 ng/lung at baseline to a peak of 0.061 ± 0.09 ng/lung on the ninth day of daily aerosol allergen challenge. Increased immunoreactivity to 15-F_{2t}-IsoP (8-iso-PGF_{2α}) or to isoketal protein adducts was found in epithelial cells 24 h after the first aerosol challenge and at 5 days in macrophages. Collagen surrounding airways and blood vessels, and airway and vascular smooth muscle, also exhibited increased immunoreactivity after ovalbumin challenge. Dietary vitamin E restriction in conjunction with allergic inflammation led to increased whole lung F₂-isoprostanes while supplemental vitamin E suppressed their formation. Similar changes in immunoreactivity to F₂-isoprostanes were seen. Airway responsiveness to methacholine was also increased by vitamin E depletion and decreased slightly by supplementation with the antioxidant. Our findings indicate that allergic airway inflammation in mice is associated with an increase in oxidant stress, which is most striking in airway epithelial cells and macrophages. Oxidant stress plays a role in the production of airway responsiveness.

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Introduction

Asthma is a chronic inflammatory disorder of the airways that is characterized by bronchial hyperresponsiveness to a variety of stimuli. A growing body of evidence suggests that oxidative stress is involved in airway inflammation in asthma [1,2]. Direct measurement of oxidants is difficult because they are highly reactive, short-lived species. Thus, oxidative stress is often measured by observing the damage inflicted by oxygen

radicals on various biomolecules, such as lipids, proteins, and deoxyribonucleic acid.

Measurement of isoprostanes (F₂-IsoPs) has emerged as one of the most reliable approaches to assess oxidative stress status in vivo [3]. The F₂-IsoPs are prostaglandin F₂-like compounds formed nonenzymatically in vivo by free radical-induced peroxidation of arachidonic acid via H₂-isoprostane intermediates. We have shown that the excretion of the major urinary metabolite of 15-F_{2t}-IsoP (8-iso-PGF_{2α}) increases significantly following inhaled allergen challenge in patients with mild atopic asthma [4]. We have also found increased levels of F₂-IsoPs in bronchoalveolar lavage fluid 24 h after segmental allergen challenge in atopic asthmatics [5]. Montuschi and colleagues demonstrated that the increased levels of F₂-IsoPs in exhaled breath condensate from patients with asthma correlate with disease severity [6]. In addition to being important markers of

Abbreviations: BAL, bronchoalveolar lavage; F₂-IsoPs, F₂-isoprostanes; GC-NICI-MS, gas chromatography/negative-ion chemical ionization mass spectrometry; IL, interleukin; PBS, phosphate-buffered saline; HRP, horseradish peroxidase; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight; IFN, interferon.

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lipid peroxidation, F₂-IsoPs can also exert potent biological activities, some of which are relevant to pathobiology in the lung [7,8]. For example, 15-F_{2t}-IsoP has been shown to induce airway hyperresponsiveness in mouse lung [9], cause dose-dependent vasoconstriction, and bronchoconstriction in isolated perfused lungs of rats [10] and guinea pigs [11], and constrict human and guinea pig bronchial smooth muscle in vitro [12]. F₂-IsoPs can stimulate the production of growth factors such as transforming growth factor- β [13], which may have relevance for airway remodeling.

We have discovered that isoprostane endoperoxide intermediates undergo rearrangement to form a series of highly reactive γ -ketoaldehyde levuglandin-like compounds termed isoketals [14–16]. These extremely reactive molecules form covalent adducts with the ϵ -amino group of lysine residues within seconds in vitro and exhibit marked proclivity to form protein-protein and protein-DNA crosslinks. These properties suggest that isoketals could significantly alter many structural and functional aspects of proteins near the sites of lipid peroxidation. Although, in vivo levels of these isoketals have not been explored extensively, conditions that increase F₂-IsoPs or prostaglandins are expected to increase formation of these isoketals [17]. We have recently shown that isoketal adduct formation can be demonstrated by immunohistochemical techniques in cells undergoing oxidant stress in vivo [18]. Therefore, like 15-F_{2t}-IsoP, isoketal adducts may serve as useful markers to elucidate the sites of lipid peroxidation and oxidative stress.

To localize the formation of F₂-IsoPs, and to attempt to determine their role in allergic inflammation, we utilized a well-characterized mouse model of allergic inflammation. We sought to modulate the formation of F₂-IsoPs by supplementing or restricting the dietary lipid chain breaking antioxidant, vitamin E. We localized lipid peroxidation to epithelial cells, to macrophages, and to lung collagen. Reduced dietary intake of vitamin E increased F₂-IsoPs immunoreactivity in epithelial cells, macrophages, and whole lung formation of F₂-IsoPs, and also altered airway responsiveness. We suggest that the free radical-catalyzed formation of F₂-IsoPs occurs during allergic inflammation and plays a role in airway responsiveness that could be relevant to human asthma.

Materials and methods

Mice

Pathogen-free 8-week-old female BALB/c mice and C57Bl/6 IL-5 knockout mice were purchased from Jackson Laboratories, Bar Harbor, Maine. They were housed in a HEPA-filtered Duo-flo laminar flow unit. Room temperature (RT) was maintained at 27°C and a 12-h-on, 12-h-off light cycle was provided. In caring for animals, the investigators adhered to the *Guide for the Care and Use of Laboratory Animals* prepared by the Committee on Care and Use of Laboratory Animal of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication No 86-23, revised 1985).

Allergen sensitization protocol

Mice received intraperitoneal injections of 0.1 ml (10 μ g) ovalbumin (chicken OVA, grade V, Sigma Chemical Co., St. Louis, MO) complexed with 20 mg of Al(OH)₃ on Day-14. On Day 0 through Day 7, the mice were placed in an acrylic box and exposed to aerosols of 1% ovalbumin diluted in sterile PBS using an ultrasonic nebulizer (Ultraneb 99, DeVilbiss, Somerset, PA) for 40 min each day.

Study protocol

Mice were fed a control diet, or one restricted or supplemented with vitamin E (synthetic, DL- α -tocopheryl acetate) (Harlan Tek-Lad) for at least 10 weeks prior to sensitization. Mice were euthanized at Day 0 and at 1 h, 24 h, Day 5, Day 9, Day 14, and Day 28 after aerosolized allergen challenge for either measurement of F₂-IsoPs by gas chromatography/negative-ion chemical ionization mass spectrometry (GC-NICI-MS) or immunohistochemical and immunofluorescence studies. Five to six mice in each group were sacrificed at each time point. Serum and lung tissue were obtained for measurement of vitamin E by HPLC. Total IgE was measured in serum. Airway responsiveness to intravenous methacholine was measured in 9–16 mice at various times as previously described [19].

Measurement of lung cytokines

Levels of IL-4, IL-5, IL-13, and IL-6 in lung tissues of the four groups of mice were measured with commercially available enzyme-linked immunosorbent assay kits (IL-4, IL-5, and IL-6 from Endogen [Woburn, MA] and IL-13 from R&D Systems [Minneapolis, MN]) according to the manufacturer's protocols [20]. On Days 3, 5, and 9 the lungs from four mice in each group were analyzed for cytokine levels. Briefly, one lung from each mouse was ground, using a mortar and pestle and ground glass. The suspension of ground lung and ground glass was then centrifuged at 800g for 10 min. The supernatant was then either frozen for later use or added to precoated wells and incubated for 2 h. Dilutions of recombinant cytokine were included for generation of a standard curve. Peroxidase-labeled anti-cytokine antibody was added to detect bound cytokine, and the plates were developed by the addition of tetramethylbenzidine substrate. Concentrations of cytokines in the lung supernatants were calculated from the standard curve produced. The cytokine level from each lung was measured in duplicate.

Measurement of F₂-IsoPs by GC-NICI-MS

For measurement of F₂-IsoPs, mouse lungs were perfused with saline to remove circulating cells and plasma, dissected free from the chest, placed immediately in phosphate-buffered saline, and ground in a tissue grinder. Folch solution containing 5 mg% BHT was added immediately and the samples

were processed. Analysis for esterified F₂-IsoPs by gas chromatography/negative-ion chemical ionization mass spectrometry was performed as previously described [5].

Vitamin E

Serum levels of vitamin E were measured by HPLC [21]. The sensitivity of the assay was 2–5 pmol/injection for α -tocopherol.

Mouse lung tissue fixation

Mouse lungs were perfused with saline through the right ventricle to remove circulating cells, inflated to 20 cm H₂O with 4% paraformaldehyde in 0.1 M PBS (pH 7.4), and fixed overnight. They were then dehydrated through graded ethanol and xylene and embedded in paraffin. Five-micrometer-thick sections were mounted on Superfrost-plus slides (Fisher Scientific, Pittsburgh, PA) and stored at room temperature until used for immunohistochemistry and immunofluorescence.

Immunohistochemical reactivity of mouse lung tissue with 15-F_{2t}-IsoP polyclonal antibody

Sections were deparaffinized and rehydrated through graded alcohols to 0.1 M PBS (pH 7.4). The sections were treated with 0.3% hydrogen peroxide for 20 min, to block endogenous peroxidase, and washed with 0.1 M PBS. Sections were incubated with 10% normal rabbit serum in 0.1 M PBS (pH 7.4) for 10 min at RT to block nonspecific binding of the secondary antibody, followed by incubation with 15-F_{2t}-IsoP antibody (Oxford Biomedical Research, MI; dilution 1:100 of 6 mg/ml protein concentration) or goat Immunoglobulin G (IgG) (Sigma-Aldrich Inc., MO), dilution 1:200 of 11.8 mg/ml protein) as a negative control, for 16 h at 4°C. The sections were then washed in 0.1 M PBS, incubated with biotinylated anti-goat secondary antibody (DakoCytomation, CA, dilution 1:400) for 1 h at RT, washed in PBS, and incubated with horseradish peroxidase (HRP) conjugated to streptavidin (Vectastain ABC kit [standard] Vector Laboratories, CA) for 30 min. The sections were again washed in PBS, incubated with 3,3-diaminobenzidine tetrahydrochloride substrate for HRP (Sigma-Aldrich Inc.) for 5 min, and rinsed with distilled water. The sections were counterstained with hematoxylin and dehydrated through graded alcohols and xylene. The sections were mounted using Cytoseal XYL (xylene based) (Stephens Scientific, NJ) and examined by light microscopy.

15-F_{2t}-IsoP polyclonal antibody blocking

The 15-F_{2t}-IsoP polyclonal antibody was coincubated with a 10-fold higher concentration of 15-F_{2t}-IsoP protein overnight at 4°C with gentle mixing. After incubation, the mixture was used for immunohistochemistry as described above. Unblocked 15-F_{2t}-IsoP polyclonal antibody was used as a positive control.

Immunohistochemical staining of mouse lung tissue with D11 (ScFv)

Tissue sections were deparaffinized, rehydrated, and treated with 0.3% hydrogen peroxide as described previously. Sections were incubated with 5% bovine serum albumin in 0.1 M PBS (pH 7.4) for 30 min at RT to block nonspecific binding of the secondary antibody. Sections were then incubated with D11 (ScFv) (5 ng/ml protein) for 2 h at RT. The sections were then washed in PBS and incubated with HRP-labeled anti-E Tag (dilution 1:500) secondary antibody for 2 h followed by treatment with 3,3-diaminobenzidine tetrahydrochloride substrate for HRP (Sigma-Aldrich Inc. The sections were counterstained with hematoxylin, dehydrated through graded alcohols and xylene, mounted using Cytoseal XL (xylene based) (Stephens Scientific), and then examined by light microscopy.

Quantitation of 15-F_{2t}-IsoP and D11 ScFv-immunoreactive cells

The following compartments of the lung were assessed: alveolar spaces, airways at all levels, interstitium, and vessels (both arteries and veins). Airway types were defined as follows: alveolar duct, walls consisting entirely of alveoli; respiratory bronchiole, occasional mural alveoli, low cuboidal epithelium; bronchiole, respiratory epithelium, no cartilage; bronchus, respiratory epithelium and cartilage. Vessels were defined as arteries if they traveled with airways and had a medial layer and veins if they were thin walled and unaccompanied by airways. In each section, airway epithelial cells were counted in 8–10 respiratory bronchioli or bronchioli (i.e., small airway) at 10× magnification and in a 210 μ m length stretch of bronchial wall (40× magnification). Similarly, for alveolar macrophages 15 fields were counted in random at 40× magnification.

BAL proteins

On Day 9 bronchoalveolar lavage samples were taken from three mice from the control diet and vitamin E-restricted diet groups. Two-dimensional electrophoresis was performed on combined paired samples after color dyeing of each sample. Using imaging software, differences in the intensity of isolated spots on the gel were determined, and analyzed by paired *t* test. Some 300 differences were found. Of these 10 were selected manually for further identification using MALDI-TOF based on consistency, size of change, and visual inspection of the individual spots.

Statistical analysis

Results were expressed as mean $\pm$ standard deviation (SE). Statistical analysis was performed using a commercial package (GraphPad Prism 3). Histologic data were compared using one-way analysis of variance with a post hoc Dunnett's test. Airway responses were compared using two-way analysis of variance with repeated measures. Statistical significance was taken when *P* < 0.05.

Results

Vitamin E levels

Restriction or supplementation of vitamin E caused substantial changes in the serum levels of vitamin E. The levels in control diet mice were 0.89 ± 0.05 :M, which were significantly different from the levels in vitamin E-supplemented (1.72 ± 0.14) or vitamin E-restricted mice (0.25 ± 0.02) ($P < 0.001$).

Measurement of F_2 -IsoPs in mouse lung

F_2 -IsoPs were measured in mouse lung at baseline and 24 h after the initial aerosol challenge with ovalbumin. Additional samples were taken on Days 5 and 9. The persistence of F_2 -IsoPs was determined by sampling at Days 14 and 28, 6 and 20 days respectively after the last aerosol challenge. In mice fed a control diet, allergen challenge provoked an increase in F_2 -IsoPs levels from baseline values of 0.30 ± 0.08 ng/lung to a peak by Day 9 of 0.61 ± 0.09 ng/lung, ($P < 0.01$) (Fig. 1). By Day 14 (6 days after the last of 8 daily aerosol challenges) the whole lung F_2 -IsoPs had returned to control values (0.24 ± 0.034 ng/lung). Mice lacking IL-5 and their congenic controls both had a roughly fourfold increase in whole lung F_2 -IsoPs levels on Day 9 over baseline (Data not shown). Consistent with the lack of IL-5, the knockout mice did not manifest an increase in eosinophils with allergen challenge.

The allergen-induced changes in whole lung F_2 -IsoPs were dramatically modified by dietary alterations in vitamin E. In the vitamin E-restricted group, the baseline F_2 -IsoPs were significantly elevated over control (0.46 ± 0.06 ng/lung) and increased threefold at Day 8 to 1.21 ± 0.20 ng/lung (Fig. 1). In marked contrast, vitamin E-supplemented diets prevented the allergen-induced increase in oxidant stress.

Immunohistochemical analysis of 15- F_2t -IsoP and isoketals

In normal mouse lung, immunohistochemical analysis revealed the presence of 15- F_2t -IsoPs and isoketal adducts in a few epithelial cells of bronchi and bronchioli, and in the collagen surrounding these structures (Figs. 2 and 3). One hour after exposure to aerosolized allergen, the numbers of epithelial cells in bronchi and bronchioli exhibiting immunoreactivity to 15- F_2t -IsoP and isoketal adducts were increased above controls. At both 1 and 24 h after the first aerosol challenge this increase was statistically significant ($P < 0.05$) (Fig. 4). The number of immunoreactive epithelial cells declined by Day 5 and had returned to control levels by Day 9, despite ongoing allergen inhalation. The positive cells in airway epithelium had the characteristic shape of Clara cells. Colocalization studies by double immunofluorescence staining using confocal laser microscopy confirmed 15- F_2t -IsoP immunoreactivity in the Clara cell (data not shown).

Altering vitamin E in the diet for 10 weeks did not affect the 15- F_2t -IsoP or isoketal adduct immunoreactivity in unsensitized animals (data not shown). Twenty-four hours after exposure to

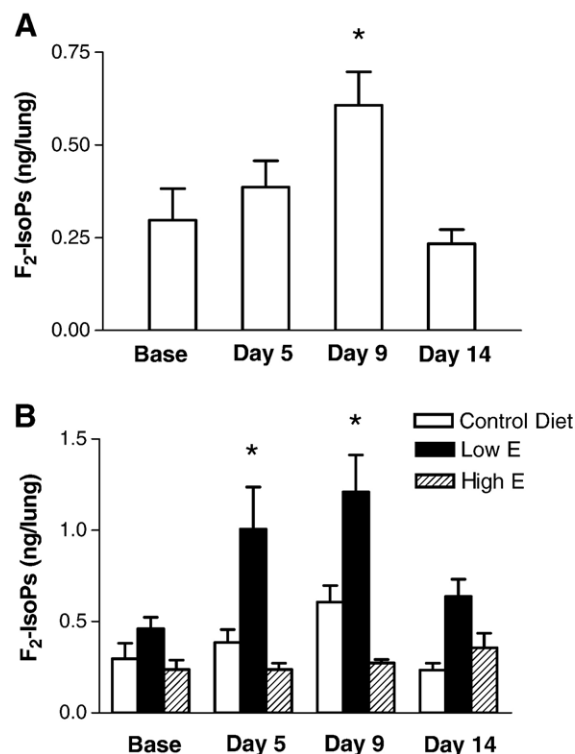


Fig. 1. (A) Levels of 15- F_2t -IsoP in ng in murine lung before and at intervals after aerosolized ovalbumin challenges in mice fed a regular diet for 10 weeks prior to experiments. Each point represents mean of 4–13 samples $\pm$ standard error. F_2 -IsoPs are increased at Day 9 (24 h after 8 daily aerosol challenges with ovalbumin) (ANOVA, $*P < 0.05$). (B) Levels of 15- F_2t -IsoP in ng in murine lung before and at intervals after aerosolized ovalbumin challenges in mice fed a regular diet (open bars), a diet lacking vitamin E (solid bars), or a diet supplemented with vitamin E (hatched bars) for 10 weeks prior to experiments. Each point represents the mean of 4–13 samples $\pm$ SE. F_2 -IsoPs are increased at Day 9 day after ovalbumin aerosol (ANOVA, $*P < 0.05$) in vitamin E-restricted and control mice, but not in mice supplemented by vitamin E. In mice fed a diet restricted in vitamin E, the levels of F_2 -IsoPs were significantly elevated above those of control diet mice and vitamin E-supplemented mice at Day 5 (24 h after 4 daily challenges) and Day 9 (ANOVA, $*P < 0.05$; post hoc Bonferroni multiple comparison test).

aerosolized antigen 15- F_2t -IsoP and isoketal adduct immunoreactivity in the vitamin E-supplemented group was less than the control group, although this did not reach a statistical significance. However, after 24 h there was a statistically significant ($P < 0.05$) increase in 15- F_2t -IsoP and isoketals immunoreactivity in the vitamin E-restricted group as compared to control group. At Day 5, there was no significant difference in control and altered vitamin E diet groups and by Day 9 15- F_2t -IsoP immunoreactivity returned to baseline.

Similarly 24 h after exposure to aerosolized allergen, there was a significant increase ($P < 0.01$) in number of 15 F_2t -IsoPs and isoketal-immunoreactive macrophages (Figs. 3 and 5). Confocal microscopy revealed that 15- F_2t -IsoP and F4/80 (a macrophage marker) colocalized in macrophages (data not shown). The number of immunoreactive macrophages increased with time and then declined (Fig. 5).

Eosinophils did not demonstrate immunoreactivity for 15- F_2t -IsoP antibody at any time point, although there was a statistically significant ($P < 0.05$) increase in the number of

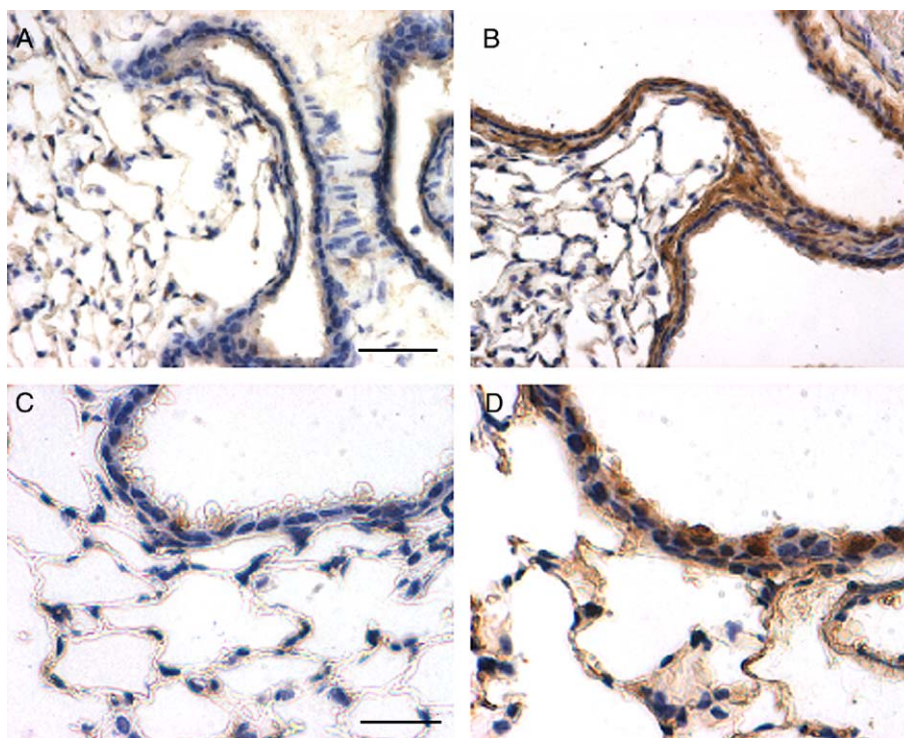


Fig. 2. Immunoreactivity to 15-F_{2t}-IsoP (A and B) (bar = 50 μ m) or isoketal adducts (C and D) (bar = 20 μ m) in murine airway epithelium 1 h after ova inhalation. The presence of the antigen is recognized by the primary antibody seen as brown. (A and C) In mice without prior i.p. sensitization, few airway epithelial cells are immunoreactive. (B and D) Sensitized and challenged mice show significant increase in immunoreactivity for both 15-F_{2t}-IsoP and isoketal adducts in airway epithelial cells and periarterial collagen.

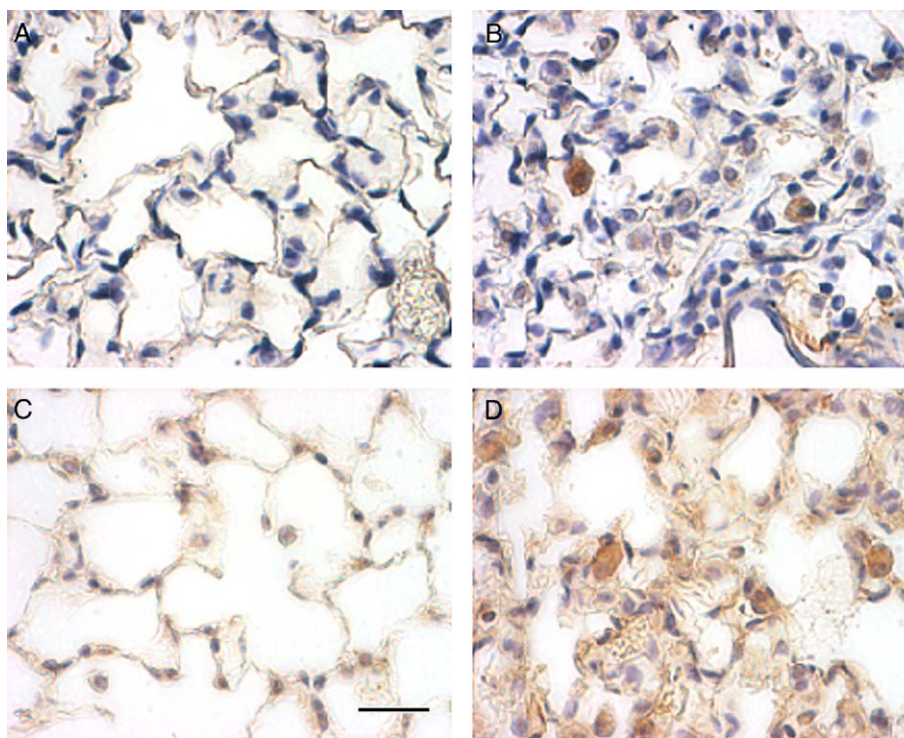


Fig. 3. Immunoreactivity of alveolar macrophages for 15-F_{2t}-IsoP (top panels) or isoketal adducts (bottom panel) on Day 5 after four daily aerosol challenges in mice sensitized with intraperitoneal ovalbumin (B and D) vs control mice (A and C). There is a significant increase in the number of macrophages immunoreactive to 15-F_{2t}-IsoP and isoketals (bar = 20 μ m).

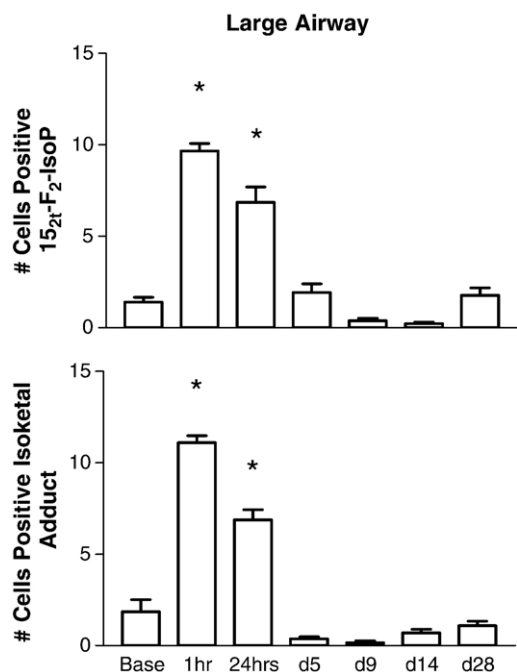


Fig. 4. The number of airway epithelial cells immunoreactive to 15-F_{2t}-IsoP (top panels) and isoketal adducts (bottom panels) in murine airway epithelium before and at intervals after aerosolized ovalbumin challenges. Height of the bars represents mean $\pm$ standard error of five animals compared statistically by ANOVA with a post hoc Dunnett test (* $P < 0.05$). 15-F_{2t}-IsoP and isoketal adducts are significantly increased at 1 and 24 h after ovalbumin aerosol.

eosinophils in the lung on Day 9 after aerosolized allergen challenge. Inhalation of ova without prior systemic sensitization resulted in no inflammatory response in the lung and no increase in immunoreactive lung F₂-IsoPs (data not shown). Preadsorbed 15-F_{2t}-IsoP demonstrated no reaction product in any cell type, in the 1-h allergen-challenged mouse lung, demonstrating the specificity of antibody.

Lung mechanics

Allergen sensitization and challenge of mice fed a regular diet resulted in an increase in responsiveness to methacholine at Days 5 and 9, with a gradual return to control values at Day 21. Altering vitamin E in the diet for 10 weeks did not affect the methacholine responses of unsensitized animals. After allergen at Day 5, the reactivity of the vitamin E-restricted group was less than that of control and the vitamin E-supplemented group. At Day 9, the vitamin E-restricted group had a significantly greater response to methacholine than the control diet group (Fig. 6). The vitamin E-supplemented group had slightly less reactivity than the control group. At Day 15, the vitamin E-supplemented group had significantly less responsiveness than the control group (Fig. 7).

Lung cells and cytokines

Eosinophils were predominant in the bronchoalveolar lavage fluid after allergen challenge. The percentage of eosinophils in BAL at Day 9 revealed no differences in the vitamin E-manip-

ulated groups (control 56 ± 7 , vitamin E restricted, 65 ± 4 , vitamin E supplemented, 59 ± 6) compared with control. Serum IgE levels did not differ among the three groups of allergen challenged mice (control diet 8.0 ± 1.9 ng/ml, vitamin E restricted 7.8 ± 1.7 , vitamin E supplemented 8.2 ± 0.8). Whole lung cytokine measurements revealed that both vitamin E-supplemented and -restricted diet mice had lower IL-4 and 5 levels at Day 3 than control mice (Fig. 8). IL-13 was similar among the three diet groups, differing only in the lower level in the vitamin E-supplemented group at Day 5. IL-10 and IFN- γ levels were undetectable at all the studied times.

BAL proteins

On Day 9 the vitamin E-restricted group had increased levels of complement 5 fragments, gelsolin, pulmonary surfactant-associated protein D and A, small inducible cytokine B15 (lung kine), and acidic mammalian chitinase. A reduced amount of vitamin D-binding protein was found compared to control diet mice.

Discussion

We have demonstrated that F₂-IsoPs are generated under normal physiological conditions in mouse lung and that levels increase significantly during allergen-induced inflammation (Fig. 1). After allergen challenge, the levels of F₂-IsoPs slowly

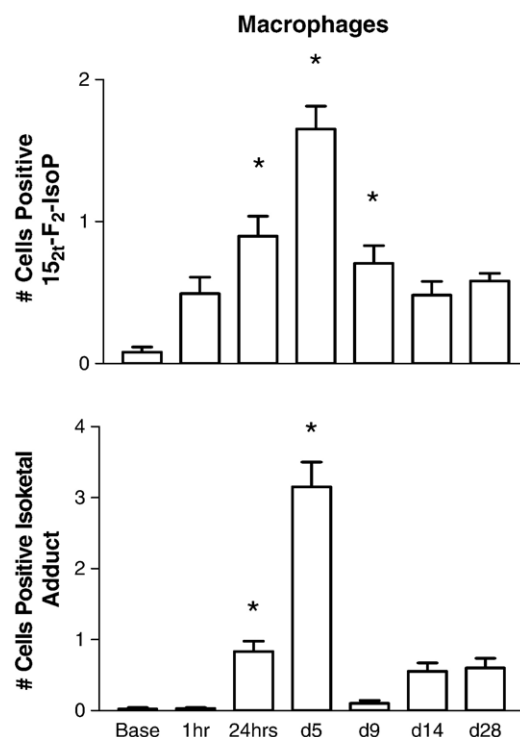


Fig. 5. The number of alveolar macrophages immunoreactive to 15-F_{2t}-IsoP and isoketal adducts before and at varying time points after aerosolized ovalbumin challenge. Height of the bars represents mean $\pm$ SD of five animals compared statistically by two-way ANOVA followed by Duncan's test (* $P < 0.05$). 15-F_{2t}-IsoP and isoketal adducts are significantly increased at Day 5 (24 h after 4 daily ovalbumin aerosol challenges).

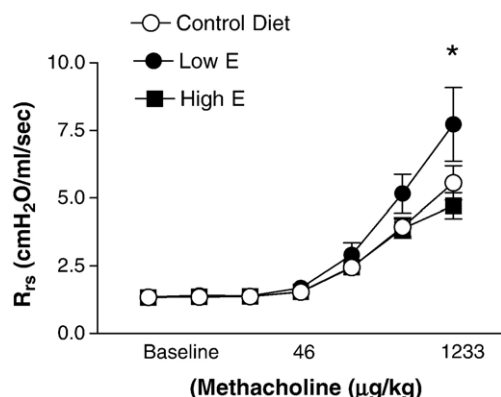


Fig. 6. Respiratory system resistance in response to tripling doses of intravenous methacholine 24 h after 8 daily ovalbumin aerosol challenges in 14 mice on control diet (open circles), vitamin E restricted (solid circles), and vitamin E supplemented (solid rectangles). Mice with dietary restriction had greater responsiveness than control or E-supplemented mice ($P < 0.05$).

increased with time, becoming statistically significant at Day 9 (after 8 days of allergen aerosols). After cessation of allergen exposure, the levels declined reaching that of nonchallenged mice by Day 28. These data provide support for the notion that allergic inflammation is accompanied by oxidative stress. Immunohistochemistry revealed that in control unchallenged mouse lung, 15-F_{2t}-IsoP was present in occasional epithelial cells of bronchi and bronchioli, and in the surrounding collagen (Fig. 2). These findings are consistent with many studies showing levels of lipid peroxidation products present in living cells and animals, presumably as a result of ongoing oxidant stress associated with an oxygen-rich atmosphere, mitochondrial leakage of free radicals, etc.

After allergen challenge, 15-F_{2t}-IsoP immunoreactivity increased in airway epithelial cells within an hour after allergen challenge (Fig. 4), suggesting that F₂-IsoPs are generated rapidly in airway epithelial cells in response to allergen. On the other hand, we did not find consistent increases in total esterified F₂-IsoPs in whole lung tissue at the same time point. Presumably this results from the minor contribution of epithe-

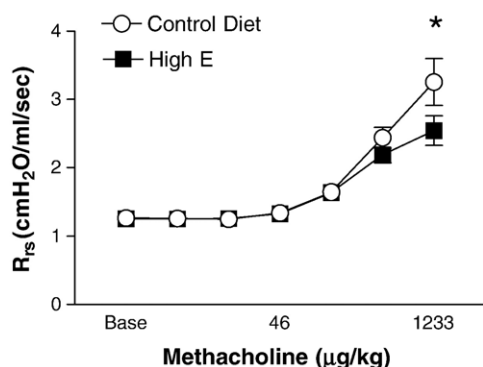


Fig. 7. Respiratory system resistance in response to tripling doses of intravenous methacholine on Day 15 (7 days after 8 daily ovalbumin aerosol challenges) in 10 mice on control diet (open circles), and vitamin E supplemented (solid rectangles). Mice with vitamin E supplements had less responsiveness than control ($P < 0.05$).

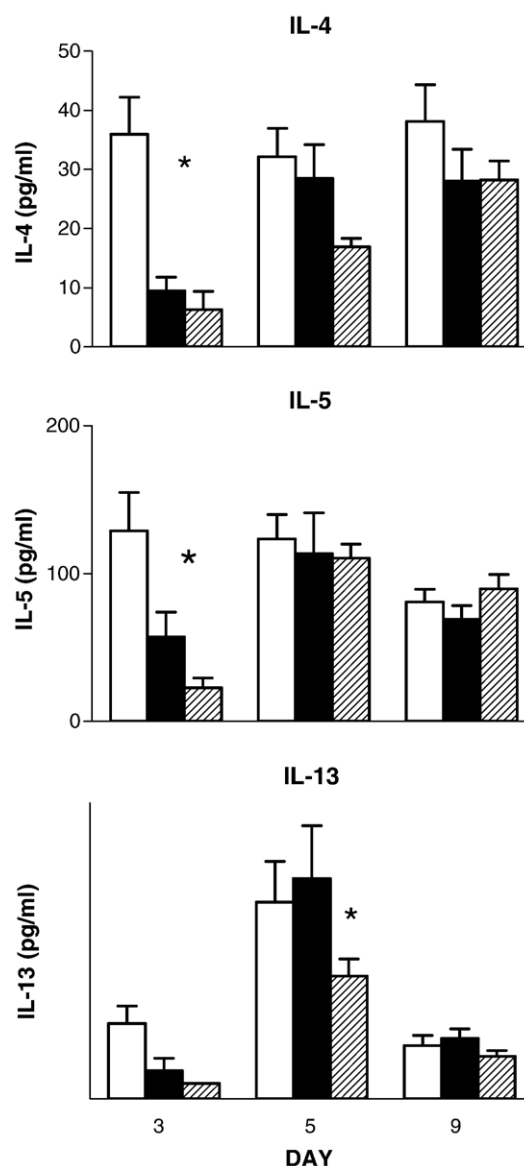


Fig. 8. Cytokine levels in whole mouse lung at Days 3, 5, and 9 (24 h after 2, 4, or 8 daily ova aerosol challenges, respectively). Height of the bars represents mean $\pm$ standard error of 3–5 lungs on control diet (open bars), vitamin E restricted (solid bars), and vitamin E supplemented (hatched bars). IL-4 and IL-5 levels were reduced compared with controls at Day 3. IL-13 was reduced at Day 5 in the vitamin E-supplemented group.

lial cell F₂-IsoPs to the total amount present in the other cells of the lung. At 9 days we suggest that there has been a global increase in tissue oxidant stress. The rapid formation of F₂-IsoPs and isoketal adducts suggests that the epithelial cell is an early target of oxidant stress during allergic reactions. Whether this is a direct effect of allergen or is mediated by inflammatory cell mediator release is uncertain. Allergen inhalation alone, without prior systemic sensitization, does not result in increased F₂-IsoPs or immunoreactivity to 15-F_{2t}-IsoP or isoketal adducts, suggesting that Clara cell oxidative stress is IgE dependent. Because bronchial epithelial cells bear IgE receptors and contain NADPH oxidase, they could become activated on crosslinking of IgE receptors, resulting in oxidant stress

[22,23]. Alternatively, certain mast cell products, including cytokines, such as IL-4 and IL-13, and enzymes, such as tryptase, have stimulatory effects on epithelial cells that could result in oxidative injury to the epithelial cell [24,25]. Eosinophils are unlikely to be involved in the stimulation of epithelial cells at this early time point, because they are not found in quantity so early after allergen inhalation.

The Clara cell is the predominant epithelial cell present in mouse airway. A plausible explanation for the decrease in epithelial cell-immunoreactive 15-F_{2t}-IsoPs with time could be a decrease in the number of Clara cells. Shijubo and associates have shown an inverse correlation between CC10 (Clara cell protein) expression in small airways of asthmatics and mast cell accumulation [26]. It is also possible that, on exposure to oxidative stress, antioxidant defenses are upregulated or allergen challenge may induce phospholipases which hydrolyze IsoPs from membranes [27].

We have also demonstrated an increase in the number of 15-F_{2t}-IsoP and isoketal adduct positive alveolar macrophages after allergen challenge (Figs. 3 and 5). Alveolar macrophages are capable of producing free radicals during activation. Previous studies have indicated enhanced production of reactive oxidant species in blood monocytes of asthmatic patients [28,29]. In asthmatic patients, nonstimulated monocytes in suspension were shown to spontaneously release greater amounts of O₂⁻ over time and release more O₂⁻ with phorbol myristate acetate stimulation, than monocytes from normal individuals. Thus macrophages activated during the course of allergen challenge could produce free radicals, leading to an increase in macrophage 15-F_{2t}-IsoP and isoketal adduct immunoreactivity. Although, macrophages are activated early in the course of allergen inhalation [30], 15-F_{2t}-IsoP and isoketal adduct immunoreactivity in macrophages was not evident until Day 5. The macrophage is a source of oxidant species, so it is perhaps not surprising that 15-F_{2t}-IsoP and isoketal adduct reactivity is present in them [31]. Alternatively, the increased number of 15-F_{2t}-IsoP and isoketal adduct-immunoreactive macrophages may arise from macrophage uptake of oxidized phospholipids and isoketal adducts shed from cell membranes.

The sources of the free radical increase during allergic inflammation are unknown. Traditional cell sources of free radicals include monocytes, macrophages, eosinophils, and neutrophils. There is controversy concerning the activation state of murine eosinophils in this model [32,33]. We did not find increased isoketal adduct or 15-F_{2t}-IsoP immunoreactivity in the infiltrating eosinophils. Moreover, oxidative stress was present in mice deficient in eosinophils because of a lack of IL-5. Thus in this murine model the eosinophils do not appear to be a major source of oxidant stress. As noted, we did observe the late appearance of F₂-IsoP and isoketal immunoreactivity in macrophages.

With alterations in the serum levels of vitamin E caused by dietary restriction or supplementation came dramatic changes in the whole lung levels of the lipid peroxide products, F₂-IsoPs. Before allergen challenge, vitamin E restriction resulted in a 50% increase in whole lung F₂-IsoPs. Allergen challenge in-

creased whole lung F₂-IsoPs even more in the E-restricted mice, while the E-supplemented mice had no allergen-induced increase in F₂-IsoPs.

In conjunction with the increase in F₂-IsoPs came an increase in airway responsiveness at Day 9 in the E-restricted group (Fig. 6) and a small decrease in Day 15 in the E-supplemented group (Fig. 7). BAL (but not tissue) eosinophils were reduced at Day 5 in the E-restricted mice. The Th2 lung cytokines IL-4, 5, and 13, did not differ from control diet mice at Day 5 or 9 in the E-manipulated groups (Fig. 8), but at Day 3, IL-4 and 5 were lower in both E groups than in control. Interferon- γ and IL-10 were not detected at any time point. Thus there was no substantial change in cytokines that might have explained the alterations in airway responsiveness. Despite the profound alteration in F₂-IsoPs formation caused by vitamin E, no change in the Clara cell or macrophage staining for F₂-IsoPs was found at baseline.

In exploring the possible mechanisms by which vitamin E restriction (and presumably, oxidant stress) could cause the increase in airway responsiveness at Day 9, we discovered that a number of bronchoalveolar lavage proteins were elevated in the vitamin E-restricted group, including lungkine, a cytokine associated with neutrophil recruitment [34], and mammalian chitinase [35]. Lungkine derives from bronchial epithelial cells and could contribute to neutrophilic inflammation. Acidic mammalian chitinase has been recently associated with asthma [36]. Overproduction of these two proteins could conceivably contribute to the increased reactivity seen in E-restricted mice. Also of interest was the increase in vitamin E-restricted mice of surfactant-related proteins, which have previously been shown to be elevated in allergic inflammation [37].

Our findings of oxidant stress during allergic airway inflammation, and the association of the degree of airway responsiveness with the level of oxidant stress, may have relevance for human asthma. Epidemiologic evidence has linked lower dietary vitamin E intake with asthma [38,39]. Attempts to treat asthma by supplementing vitamin E have not been wholly convincing, but in these studies typically no measures of oxidant stress were undertaken [40]. We have preliminary data suggesting that in man high doses of vitamin E (800 mg a day) are required to suppress serum F₂-IsoP levels, and that at least 14 weeks of treatment is needed to accomplish this [41]. Thus it may be that previous trials did not supplement vitamin E in sufficient doses and for a duration that would affect lipid peroxidation significantly. On the other hand, there is suggestive evidence that vitamin E could prevent the onset of asthma [42,43]. Thus the role of vitamin E in reducing oxidant stress in human asthma and its possible therapeutic effect remains uncertain.

In summary, we have localized oxidative stress by 15-F_{2t}-IsoP and isoketal adduct immunoreactivity in murine lung allergic inflammation to airway epithelial cells and macrophages. By measurement of the lipid peroxide products, F₂-IsoPs, we have demonstrated an increase in oxidant stress caused by allergic inflammation in the mouse. By manipulating the levels of the lipid chain breaking antioxidant vitamin E we could increase or suppress oxidant stress, and could affect

airway responsiveness. The change in airway responsiveness with increased oxidant stress was associated with higher levels of surfactant proteins, mammalian chitinase, and a lung-specific cytokine (lungkine [34,35]). Overall our data show that oxidant stress occurs with allergic inflammation, and that increases or decreases in the degree of oxidant stress caused by varied levels of dietary vitamin E alter airway responsiveness. These findings may have relevance for human asthma, and for the use of antioxidants in asthma treatment trials.

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