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Ozone Enhancement of Lower Airway Allergic Inflammation is Prevented By γ -Tocopherol

James G. Wagner¹, Qing Jiang², Jack R. Harkema¹, Beate Illek³, Dhavalkumar D. Patel⁴, Bruce N. Ames³, and David B. Peden⁴

¹Department of Pathobiology and Diagnostic Investigation, Michigan State University, East Lansing, MI

²Interdepartmental Nutrition Program, Department of Foods and Nutrition, Purdue University, West Lafayette, IN

³ Children's Hospital of Oakland Research Institute, Oakland, CA

⁴Center for Environmental Medicine, Asthma, and Lung Biology, University of North Carolina, Chapel Hill, NC

Abstract

Ozone is a commonly encountered environmental oxidant which has been linked to asthma exacerbation in epidemiological studies. Ozone induces airway inflammation and enhances response to inhaled allergen. It has been suggested that antioxidant therapy may minimize the adverse effects of ozone in asthma. We have previously shown that the antioxidant gamma-tocopherol (γ T), an isoform of vitamin E, also has anti-inflammatory effects. We employed a Brown Norway rat model of ozone-enhanced allergic responses to test the therapeutic effects of γ T on O₃-induced airway inflammation. Ovalbumin (OVA) -sensitized rats were intranasally challenged with 0 or 0.5% OVA on Days 1 and 2, and exposed to 0 or 1 ppm ozone (8h/day) on Days 4 and 5. Rats were also given 0 or 100 mg/kg γ T on Days 2 through 5. Pulmonary tissue and bronchoalveolar lavage fluid (BALF) were collected on Day 6. OVA challenge caused increased total cells (267% increase) and eosinophils (4000%) in BALF that was unaffected by ozone exposure. Morphometric evaluation of lung tissue revealed increases in intraepithelial mucosubstances (IM) (300%) and subepithelial eosinophils (400%) in main axial airways. Ozone exposure of allergic rats enhanced IM increases in proximal axial airways (200%), induced cys-leukotrienes, MCP-1 and IL-6 production in BALF, and upregulated expression of IL-5 and IL-13 mRNA. γ T treatment had no effect on IM increases by allergen, but blocked enhancement by ozone. γ T attenuated both OVA- or ozone -stimulated eosinophilic infiltration, and increases of BALF cys-leukotrienes, MCP-1 and IL-6, as well as IL-5 and IL-13 mRNA. These data

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Address for Correspondence: Dr. James G. Wagner 211 Food Safety and Toxicology Bldg Department of Pathobiology and Diagnostic Investigation Michigan State University, East Lansing, MI, 48824 Ph (517)432-1026 Fax (517)353-9902 wagnerja@msu.edu.

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demonstrate broad anti-inflammatory effects of a γ T and suggest it may be an effective therapy of allergic airway inflammation.

Keywords

γ -tocopherol; vitamin E=; ozone; ovalbumin; mucous cell metaplasia; eosinophil; inflammation

INTRODUCTION

Epidemiological studies implicate a role for ozone, the most prominent oxidant pollutant in photochemical smog, in the exacerbation of allergic airway diseases. Increases in ambient ozone concentration are associated with hospital admissions and reported symptoms from individuals with asthma and allergic rhinitis [1-3]. In challenge studies of asthmatic subjects, our research group has shown ozone induces eosinophilic infiltration in both upper and lower airways, decrements in pulmonary function, and enhances response to allergen [4-6]. Even in the absence of provocation with an allergen, atopic asthmatics appear to mount greater airway inflammatory responses after ozone exposure than healthy subjects [7, 8].

Specific mechanisms by which ozone exacerbates allergic responses are incompletely understood. Asthma is a disease of chronic inflammation in which an imbalance of oxidant versus antioxidant species is thought to contribute to disease severity and incidence of symptoms. Decreases in antioxidant vitamins E and C, and increases in oxidized glutathione have been reported in airway fluids of asthmatics [9-11]. In addition, enzymatic anti-inflammatory capacity in the lung and plasma of asthmatics is depressed as indicated by lower activities of glutathione peroxidase, superoxide dismutase and catalase [12-14]. Deficient defenses in the airway microenvironment could allow for increased sensitivity to further oxidative insult by ozone exposure. Deficits in the antioxidant repertoire in allergic airways may be due to a lower dietary intake of antioxidants compounds such as from fruits and vegetables, especially in persons with genetically defined decreases in antioxidant defense. In case studies, intake of vitamins C, E and carotene by asthmatics was inversely correlated with symptom severity [15-17], and asthmatics tend to have lower amounts of antioxidant nutrients in their diets, especially of vitamins E and C [18].

Although α -tocopherol (α T), the major form of vitamin E in mammalian tissues is thought to neutralize lipid radicals, and inhibit cyclooxygenase (COX) and leukotriene synthesis [19], intervention with α T supplements failed to appreciably improve allergic airway symptoms in both human and animal studies [20-22]. However, γ -tocopherol (γ T), the major form of vitamin E in US diets, has been demonstrated to be more powerful in trapping electrophiles such as reactive nitrogen species [23, 24] and displays a broader anti-inflammatory profile than α T [25]. In addition to neutralizing oxidized lipid radicals, γ T also protects both lipids and proteins from nitrosative damage from nitric oxide-derived metabolites [26, 27] and is a more potent inhibitor of COX activity than α T [28]. Furthermore, in an animal model of inflammation, we have shown that γ T is superior to α T for inhibition of key mediators of inflammation such as TNF α , nitric oxide, and inflammatory eicosanoid production [29].

We hypothesized that the γ T form of vitamin E would inhibit pollutant and allergen induced exacerbation of allergic airway inflammation. To test this hypothesis, we used a rodent model of allergic airways disease to assess the ability of γ T to prevent ozone and allergen-induced exacerbation of airway inflammation. As described herein, we observed that γ T attenuated allergen-and ozone-induced inflammatory cell recruitment, cytokine gene expression, and overproduction of mucus that characterizes asthma.

MATERIALS AND METHODS

Animals

Fifty-six male Brown Norway rats (Harlan, Indianapolis, IN), 10-12 weeks of age, were randomly assigned to one of 8 experimental groups ($n = 7$ /group). Rats were free of pathogens and respiratory disease, and used in accordance with guidelines set forth by the All-University Committee on Animal Use and Care at Michigan State University. Animals were housed two per cage in polycarbonate boxes on aspen chip bedding, covered with filter lids, and had free access to tap water and food (Tek Lad 22/5 Rodent Diet W 8649, Harlan Sprague Dawley, Indianapolis, IN). During the inhalation portion of the study, rats were housed individually in rack-mounted stainless steel wire cages in Hazelton whole-body inhalation exposure chambers (HC-100, Lab Products, Maywood, NJ). During ozone exposure, food was removed, but water was available.

γ -Tocopherol

γ -gamma-tocopherol (γ T; R,R,R naturally occurring, 96% pure by HPLC and NMR) was obtained from Yasoo Health Inc (Johnson City, TN). Stock and diluted preparations were kept under nitrogen at 4°C and contained no other tocopherols/tocotrienols or oxidation products. Prior to treatment protocols γ T was diluted in tocopherol-stripped corn oil (Dyets Inc., Bethlehem, PA).

Ozone Exposure

Ozone was generated with an OREC model O3V1-O ozonizer (Ozone Research and Equipment Corp., Phoenix, AZ) using compressed air as a source of oxygen. Total airflow through the exposure chambers was 250 L/min (15 chamber air changes/h). The concentration of ozone within the chambers was monitored throughout the exposure using two Dasibi 1003 AH ambient air ozone monitors (Dasibi Environmental Corp., Glendale, CA). Sampling probes were placed in the breathing zone of rats within the middle of the cage racks. The concentration of ozone during exposures was 0.994 ± 0.012 ppm (mean \pm SEM) for ozone chambers and less than 0.02 ppm for chambers receiving filtered air. Ozone concentrations of 1ppm were used to enhance allergic responses in a rat strain that is comparatively less sensitive to ozone than other rats or humans. Pulmonary ozone dosimetry studies demonstrate that humans have 4-5 times greater pulmonary deposition of inhaled ozone than Fisher F344 rats [30], and that rats required greater exposures for similar responses. Furthermore the Brown Norway rat is much less sensitive to ozone than F344 rats. As such, 1 ppm ozone in Brown Norway rats provides a reasonable exposure scenario to what might occur in humans.

Treatment and Exposure Protocols

The time course for treatment protocols is summarized in Figure 1. On Day 1, rats were sensitized to chicken albumin (ovalbumin; OVA; Sigma Chemical Co., St. Louis, MO) by intraperitoneal injection with 2.5 ml sterile saline of a solution containing 1 mg OVA prepared with 10 mg alum (aluminum potassium sulfate) which acts as an adjuvant. Two weeks later rats were challenged to OVA or saline for two consecutive days (i.e., Days 14 and 15) under light anesthesia (4% halothane in oxygen) by intranasal instillation (IN) with 150 μ l of 0 or 0.5% OVA in saline into each nasal passage (300 μ l total volume). IN challenge procedures were conducted at 10:00am each day.

Beginning 8h after the last IN challenge, rats were administered by oral gavage, 100 mg/kg body weight γ T prepared in tocopherol-stripped corn oil. Treatment with γ T was repeated daily at 6:00pm for four consecutive days (Days 15-18). Beginning on Day 17, animals were exposed to 0 or 1.0 ppm ozone for 8h/day (7:30am – 3:30pm) for two consecutive days (Days 17 and 18). Animals were euthanized and tissues were collected on Day 19.

Necropsy, Lavage Collection and Tissue Preparation

Animals were anesthetized with sodium pentobarbital (50 mg/kg), a midline laparotomy was performed, 4 ml of blood was drawn into a vacutainer for separation of plasma, and animals were exsanguinated by cutting the abdominal aorta. Immediately after death, the trachea was exposed and cannulated, the heart and lung were excised *en bloc*. The bronchus to the left lung was temporarily closed with a hemostatic clamp, and 4 ml of sterile saline was instilled through the tracheal cannula and withdrawn to recover bronchoalveolar lavage fluid (BALF) from the right lung lobes. A second saline lavage was performed and combined with the first.

After lavage, the right lung lobes were ligated and removed. The axial conducting airway from the right caudal lobe was removed by microdissection and homogenized in 0.5 ml RNAlater (Qiagen Inc., Valencia, CA) using a post-mounted homogenizer with a 5-mm generator (Model 250, Pro-Scientific, Inc., Monroe, CT). The right cranial lobe was also excised and placed in RNAlater. Samples were kept at -80°C until further processing for RNA isolation. The clamp was removed from the left bronchus, and the left lobe was inflated under constant pressure (30 cm H_2O) with neutral-buffered formalin for 2h, while immersed in a large volume of fixative. Twenty-four hours later, two sections were excised at the level of the 5th and 11th airway generation along the main axial airway (G5 and G11), to sample proximal and distal airways, respectively (Harkema and Hotchkiss, 1992).

Tissue blocks were then embedded in paraffin, and 5-6 μm thick sections were cut from the anterior surface. Lung sections were stained with hematoxylin and eosin (H&E) for routine histology, with Alcian Blue (pH 2.5)/Periodic Acid-Schiff (AB/PAS) to detect intraepithelial mucosubstances, or with May Grunwald stain to detect eosinophils.

Bronchoalveolar Lavage Fluid (BALF)

Cellularity—Total leukocytes in BALF were enumerated with a hemocytometer, and fractions of eosinophils, neutrophils, macrophages, and lymphocytes were determined in a cytospin sample stained with Diff-Quick (Dade Behring, Newark, DE).

Cytokine and Leukotriene Content—For leukotriene analysis, cell-free BAL fluid was first purified in 2mL methanol and 5mL hexane to precipitate proteins and lipid, respectively. The methanol layer containing leukotrienes was dried under N₂ stream. The amount of LTB₄ and total cysteinyl-leukotrienes (LTC₄, LTD₄ and LTE₄) were measured using an EIA assay from Cayman Chemicals (Ann Arbor, MI). Cytokine content in BAL fluid was determined using a custom bead-based multiplex assay capable of detecting 15 different rat protein targets. Assays are conducted in 96-well MultiScreen plates (Millipore) by the same principle of standard ELISA. Bound protein:antibody conjugates on beads are detected using a Streptavidin-PE system and analyzed using the Luminex¹⁰⁰ total system (Luminex Corp, Austin, TX).

Tocopherols—Plasma and BAL fluid α T and γ T were extracted using a mixture of methanol/hexane (2:5, v/v) in the presence of 0.8 mM butylated hydroxytoluene (BHT). After brief centrifugation at 4°C, the top hexane layer was dried under N₂ and the residue was resuspended in ethanol. Tocopherols were separated on a 150 × 4.6 mm, 5 μ m Supelcosil™ LC-18-DB column (Supelco, Bellefonte, PA, USA) and eluted with 95:5 (v/v) methanol/0.1M lithium acetate (final 25 mM, pH 4.75) at a flow rate of 1.3 mL/min. Tocopherols were monitored by coulometric detection (Model Coulochem II, ESA Inc., Chelmsford, MA, USA) at 300 (upstream) and 500 mV (downstream electrode) using a Model 5011 analytical cell.

Real Time RT-PCR

Total RNA was extracted using Rneasy Mini Kit according to manufacturers instructions (Qiagen, Valencia, CA). The evaluation of relative expression levels of IL-5 and -13 mRNA was performed by two-step RT-PCR using manufacturer protocols (Applied Biosystems, Foster City, CA). Briefly, reverse transcription was performed using High Capacity cDNA archive Kit reagents. Quantitative mRNA expression analysis was performed using an ABI PRISM 7900 HT Sequence Detection System at Michigan State University's Research Technology Support Facility using Taqman Gene Expression Assay reagents (Applied Biosystems). Relative gene expression was normalized to 18S. Data is expressed as fold-increase in RNA expression compared to control animals, which are set at a value of 1.

Morphometry of Stored Intraepithelial Mucosubstances (IM)

To estimate the amount of the intraepithelial mucosubstances (IM) in respiratory epithelium lining pulmonary axial airways, the volume density (V_s) of AB/PAS-stained mucosubstances was quantified using computerized image analysis and standard morphometric techniques. The area of AB/PAS stained mucosubstance was calculated from the automatically circumscribed perimeter of stained material using the public domain NIH Image program (written by Wayne Rasband, U.S. National Institutes of Health and available on the Internet at <http://rsb.info.nih.gov/nih-image/>). The length of the basal lamina

underlying the surface epithelium was calculated from the contour length of the digitized image of the basal lamina. The volume density (V_s) of intraepithelial mucosubstances is expressed as nanoliters of intraepithelial mucosubstances per mm^2 of basal lamina.

Morphometry of Tissue Eosinophil Numeric Density

The numeric density of eosinophils in the mucosa underlying the respiratory epithelium of conducting pulmonary airways, were determined by counting May-Grunwald-positive stained cells with distinct eosinophil morphology. The length of the basal lamina was calculated from its contour length in a digitized image as described above, and the data is expressed as the number of eosinophils divided by the length of the underlying basal lamina.

Statistical Analysis

Data are expressed as mean \pm standard error of the mean (SEM). Data were analyzed using a completely randomized analysis of variance. Multiple comparisons were made by Student-Newman-Keuls *post hoc* test. Criterion for significance was taken to be $p < 0.05$.

RESULTS

Tocopherol Content in Plasma and BALF

γ T was not detected in BALF from corn oil vehicle treated rats regardless of ozone exposure or allergen challenge (Figure 2A). Administration with γ T produced measurable levels of this tocopherol in all BALF samples, but there were no differences in any treatment group compared to saline/air controls. α T was detected in all BALF samples (Figure 2B). Treatment related changes were detected only in ozone-exposed allergic rats given 0 mg/kg γ T (Figure 2B), while apparent increases in similarly ozone-treated rats given 100 mg/kg γ T were not statistically significant ($p=0.053$).

Plasma γ T was unaffected by any exposure or challenge regimens in control, corn oil-fed rats, but was increased in all groups 4 to 5-fold by administration with 100 mg/kg γ T. No differences were detected between exposure or challenge groups (Figure 2C). Plasma α T was similar in all groups given 0mg/kg γ T, with trends toward decreases in OVA-challenged rats (Figure 2D). Allergic animals dosed with 100mg/kg γ T and exposed to filtered air had significantly less α T in plasma than saline-challenged, air-exposed rats.

Histopathology

Rats instilled with saline and exposed to ozone—No exposure related lesions were found in the lungs of filtered air-exposed rats that were intranasally instilled with only saline. In contrast, repeated ozone exposures induced site-specific lung lesions that were restricted to centriacinar regions (junction of terminal bronchioles and proximal alveolar ducts) throughout the lung lobe (Figure 3E). These centriacinar lesions were characterized by a conspicuous thickening of the walls of terminal bronchioles and proximal alveolar ducts with associated small aggregates of alveolar macrophages/monocytes and lesser numbers of neutrophils, eosinophils and lymphocytes in the alveolar airspaces of the proximal alveolar ducts. Intramural thickening was mainly due to 1) a mild-moderate influx of mixed inflammatory cells consisting of neutrophils, eosinophils, and mononuclear

inflammatory cells (lymphocytes and monocytes) and 2) an associated hypertrophy and hyperplasia of cuboidal epithelial cells lining the centriacinar airways (Figure 3E). Mucous cell hyperplasia/metaplasia, a principal feature in the conducting airway epithelium of ovalbumin-instilled rats, was not present in the large- and small-diameter conducting airways of these saline-instilled and ozone-exposed rats. The alveolar parenchyma outside of the centriacinar regions resembled that of the control rats instilled with saline and exposed only to filtered air (0 ppm ozone). Interestingly, treatment with γ T did not alter the morphologic character or severity of the centriacinar lesions caused by the ozone exposures (Figure 3F).

Rats instilled with ovalbumin (OVA) and exposed to air alone or ozone—

Intranasal challenge of OVA in filtered air or ozone-exposed rats induced an allergic bronchiolitis of the intrapulmonary conducting airways involving both large-diameter, proximal, axial airways and small diameter, distal, preterminal and terminal bronchioles. The OVA-induced inflammatory and epithelial changes in the conducting airways within the proximal transverse section of the left lung lobe (axial airway level G5) were more marked than those in the distal lung lobe section (axial airway level G11), indicating a proximal-distal decrease in the lobar lesion severity and distribution. OVA-induced bronchiolitis was characterized by peribronchiolar edema associated with a mixed inflammatory cell influx of lymphocytes, plasma cells, eosinophils, and occasional neutrophils (Figure 3C). Peribronchiolar inflammation was principally located in the subepithelial interstitial tissues (e.g., lamina propria and submucosa) with markedly fewer inflammatory cells in the surface epithelium that lines these airways. Bronchiole-associated lymphoid tissues in these OVA-challenged airways were also enlarged due to lymphoid hyperplasia. Perivascular interstitial (i.e., surrounding pulmonary arteries/arterioles adjacent to bronchioles and pulmonary veins scattered throughout the alveolar parenchyma) accumulation of a similar mixture of eosinophils and mononuclear cells, along with perivascular edema, were also present in the lungs of OVA-challenged rats (Figure 3E).

In addition to the perivascular and peribronchiolar lesions, there were varying sized focal areas of allergic alveolitis in the parenchyma surrounding or adjacent to the affected large and small conducting airways. These alveolar lesions were characterized by accumulations of large numbers of alveolar macrophages, epithelioid cells, and eosinophils, with lesser numbers of lymphocytes, monocytes and plasma cells, in the alveolar airspace. Often the alveolar septa in these areas of alveolitis were thickened due to mild-to-moderate alveolar type II cell hyperplasia/hypertrophy, intracapillary accumulation of inflammatory cells, and capillary congestion.

OVA-challenged rats exposed to either filtered air or ozone had conspicuous hypertrophy (tall columnar epithelial cells) and mucous cell metaplasia/hyperplasia (MCM) with increased amounts of AB/PAS-stained intraepithelial mucosubstances (IM) in the respiratory epithelium lining the large-diameter intrapulmonary airways, including the proximal axial airway (G5) and to a much lesser extent in the distal axial airway (G11) and the small-diameter preterminal and terminal bronchioles (Figure 4B, E). In contrast, saline-instilled rats exposed to either filtered air (Figure 4A, D) or ozone had significantly fewer mucous cells and IM in a much thinner, low columnar, respiratory epithelium lining large-diameter

airways. Very few or no mucous cells with only scant amounts of IM were present in the respiratory epithelium lining small diameter preterminal bronchioles of these saline-instilled animals. Terminal bronchioles of saline-instilled rats contained no mucous cells. Interestingly, epithelial and inflammatory lesions observed in the centriacini of ozone-exposed rats intranasally instilled with only saline, described above, were not microscopically detectable in the ozone-exposed, OVA-challenged rats, even in those centriacinar regions with little or no OVA-induced lesions (Figure 3G).

Treatment with γ T markedly attenuated OVA-induced epithelial and inflammatory lesions in both the intrapulmonary proximal and distal conducting airways and in the alveolar parenchyma (Figure 3D). Airway epithelial thickening with MCM, peribronchial and perivascular inflammatory cell influx, and alveolitis were all markedly reduced both in severity and distribution in the lungs of OVA-challenged rats that were exposed either to filtered air alone or ozone (Figures 3,4). Surprisingly, centriacinar inflammatory and epithelial lesions similar to those observed in the saline-instilled rats exposed to ozone were also present in the OVA-challenged, ozone-exposed rats that received γ T, indicating that γ T treatment was effective in attenuating allergen- but not oxidant-induced pulmonary injury in Brown Norway rats (Figure 3H).

Bronchoalveolar Lavage Fluid (BALF)

Cellularity—Ozone exposure of saline-challenged rats had no effect on lavage cellularity (Figure 5). Conversely, provocation of allergic rats with OVA induced significant accumulation of total cells, eosinophils, and macrophages in BALF, that was unaffected by ozone exposure. However ozone exposure of OVA-challenged animals caused increases in BALF neutrophils compared to OVA-challenged rats exposed to filtered air (Figure 5). γ T supplementation led to a reduction of OVA- and ozone-induced increases in BALF total cells, eosinophils, and macrophages, but not neutrophils. Furthermore, neutrophils were significantly greater in saline-challenged, ozone-exposed animals given γ T compared those dosed with corn oil.

Leukotrienes and Cytokines—Ozone exposure or OVA challenge alone had no effect on cysteinyl leukotrienes (Cys-LTs) levels in BALF (Figure 6A). However ozone exposure to allergic rats increased Cys-LTs by 3-4 fold over controls, which was blocked completely by γ T supplementation. Increases in BALF LTB₄ in allergic rats were not statistically significant, irrespective of ozone exposure (Figure 6B). γ T caused a reduction of LTB₄ in allergic rats exposed to ozone, compared to similarly treated rats given corn oil.

BALF MCP-1 was unaffected by ozone exposure alone but was increased 4-fold in OVA-challenged rats (Figure 6C). Ozone exposure further enhanced MCP-1 in allergic rats by another 3-fold. Only allergic rats exposed to ozone had significant amounts of IL-6 in BALF. Both ozone-induced responses in BALF MCP-1 and IL-6 were attenuated by supplementation with γ T.

Morphometry

Mucous Cell Metaplasia and Eosinophilic Inflammation—Two consecutive days of ozone exposure did not alter the amount of IM in pulmonary airways (Figure 7A & B). Challenge with OVA caused mucous cell metaplasia (i.e., increased IM) in the respiratory epithelium lining distal pulmonary axial airways (Figure 7B), while apparent increases in IM in proximal airways were not statistically significant (Figure 7A). However, ozone exposure caused IM levels to be significant in proximal airways of allergic rats, with a trend toward further increases in distal airways ($p=0.21$). Treatment with γ T attenuated the increases in IM in both proximal and distal airways of ozone-exposed allergic rats. By contrast, OVA-induced increases in IM remained elevated in γ T-treated, OVA-challenged rats exposed to filtered air.

OVA challenge induced a 4- to 5-fold increase in the number of eosinophils in the submucosa surrounding proximal and distal axial airways (Figures 7C & D). Ozone exposures induced a non-significant trend for increased eosinophil accumulation in allergic rats. In both air- and ozone-exposed allergic rats given γ T, eosinophil numbers were attenuated in both proximal and distal airways.

Gene Expression

Allergic rats exposed to ozone were characterized by higher expression levels of inflammatory genes IL-5 (1.9-fold) and IL-13 (2.9-fold) in the cranial lung lobe (Figure 8). Changes induced by allergen alone were not statistically different from those detected in control animals. Supplementation with γ T inhibited ozone-induced changes in gene expression for both IL-5 and IL-13.

DISCUSSION

This study was designed to determine if γ T inhibited ozone-induced exacerbation of allergic inflammation in a rodent model of allergic inflammation. Our models using the allergic Brown Norway rat mimics the human clinical condition in which asthma exacerbations are induced by ozone exposure. In the present protocol, we generally found that ozone alone had little effect on airway inflammation of OVA-sensitized rats, but challenge with OVA alone or OVA plus ozone had remarkable effects on many elements of allergen-induced inflammation. Intriguingly, ozone did not appear to augment cellular responses to OVA (eosinophil influx), but dual exposure to both agents was necessary to induce increases in inflammatory mediators, gene expression and mucous cell metaplasia.

We found that short-term acute supplementation with γ T inhibited inflammatory changes in airways of OVA-sensitized rats, whether they were challenged with OVA alone, or with OVA coupled with ozone exposure. Dosing with γ T attenuated inflammatory cell recruitment, airway cytokine and leukotriene production, epithelial cell metaplasia, mucous storage, and gene expression. It is noteworthy that the anti-inflammatory effects of γ T were observed with acute dosing that occurred after acute challenges with allergen. Thus, it was not necessary to supply γ T for several days prior to challenge, suggesting that rapidly induced serum changes in γ T, rather than chronic changes in tissue levels of γ T or its

metabolites were sufficient to protect against inflammatory changes due to ovalbumin or ozone. These novel effects suggest that γ T might be an affective adjunct or complementary intervention for asthma.

We used pharmacological doses of 100mg/kg γ T in rodents, which extrapolates to about 6-times the human recommended upper limit for the alpha-form of Vitamin E. However preliminary data in our laboratory suggests that antiallergic effects of γ T may occur as low as 10mg/kg (data not shown). Although the doses appropriate to humans need to be established, the bioavailability of γ T in human tissues are much higher than that in rodents; concentrations of γ T in human skin, adipose and muscle tissue are 6-60 fold greater than those in laboratory rodents with similar γ T intake [31, 32].

We designed our protocols for γ T pretreatment to specifically target airway responses to ozone exposure by dosing with γ T after initiating allergic inflammation, but before beginning exposures to ozone. One of our striking findings however, is that γ T specifically inhibited allergen-induced responses, despite being administered 10 hours after the second of two airway provocations with allergen. OVA-induced increases in BALF eosinophils and macrophages (Figure 5), MCP-1 (Figure 6), and eosinophilic inflammation surrounding conducting airways (Figure 7), were all attenuated by γ T supplementation in air-exposed animals. Furthermore the extent and severity of pulmonary lesions in allergic lungs, including interstitial edema and the involvement of both bronchiolar and alveolar airways, were markedly decreased in rats given γ T. These data demonstrate that acute dosing with γ T can arrest ongoing pulmonary inflammation that is downstream from the initial allergic stimulus and does not require weeks of dietary intake to produce therapeutic effects. By contrast, the present protocols did not test if γ T could prevent responses from the initial interactions of airway allergen, such as uptake by dendritic cells or IgE-mediated activation of alveolar macrophages and mast cells. The possible prophylactic effects of γ T on allergic responses are currently under investigation.

In addition to blocking responses engendered by OVA provocation, γ T also attenuated all ozone-induced enhancements of allergic responses. Under the current experimental design, γ T was administered twice by the time of the first ozone exposure, and administered immediately after each exposure (Figure 1). This acute supplementation regimen was apparently sufficient for pulmonary γ T to block any acute oxidant or inflammatory stimuli induced by inhalation with ozone. We have previously described the requirement for neutrophils in the initiation and progression of ozone-induced MCM in a non-allergic model [33], as well as associations of eosinophils and neutrophils with MCM in allergic models [34]. In the present study, eosinophilic infiltrates that were dramatically increased in BALF by allergen were attenuated by supplementation with γ T. We also saw a trend for ozone-induced enhancement of tissue eosinophils that was inhibited by γ T. Although ozone did not dramatically enhance eosinophil numbers in allergic rats, ozone (or mediators generated after ozone exposure) may activate inflammatory cells that infiltrate tissues after allergen challenge to release factors that promote MCM. This idea is supported by our observation that ozone exposure coupled with OVA challenge (but neither stimulus alone) led to a significant increase in cysteinyl leukotrienes (cysLTs), MCP-1, and IL-6 as indicated by their increases in BALF (Figure 6). These mediators were not present in allergic animals

exposed to filtered air, despite significant infiltration of inflammatory cells into lung tissue. Ozone promotes lipoxygenase activity in the lung [35], and cysLTs have been shown to induce MCM in allergic rodents [36, 37]. Eosinophils and IgE-activated mast cells are primary sources of cysLTs in allergic airways. That ozone can activate both mast cells [38] and eosinophils [39] in healthy airways, suggests it may promote allergic MCM by stimulating eosinophils, mast cells and other inflammatory or epithelial cells for enhanced production of inflammagens, including cysLTs. Complete blockade by γ T of BALF cysLTs agrees with our previous finding of LOX inhibition by γ T [29]. Taken together, the therapeutic effects of γ T in ozone-exposed allergic rats may be due to inhibitions of OVA-initiated inflammatory cell recruitment and ozone-induced activation of inflammatory and epithelial airway cells.

Interestingly, γ T was effective at resolving the ongoing responses of inflammatory cell infiltration (Figure 5), but had no effect on allergic mucous cell metaplasia (Figure 7). These differential inhibitory effects might be explained by the acute versus chronic stimuli elicited by 1) acute allergen exposure, and then 2) the cascade of cellular and soluble mediators engendered over a period of hours and days. Recurring signals for cell recruitment and activation persist well after the initial allergen stimulus, and are still present when γ T dosing begins. In contrast, metaplastic changes to airway epithelium (e.g., mucous cell metaplasia) are a relatively long-lived phenotypic alteration that is initiated by signaling pathways present early after an allergen challenge (e.g., 6-48h). These early pathways of mucin gene expression and metaplasia have been shown to involve intracellular reactive oxygen species [40, 41], production of which during our protocol would likely occur well before supplementation with γ T. Thus, interruption by γ T of allergic inflammation versus its lack of effect on allergic mucous cell metaplasia may be dependent on the timing of γ T supplementation during allergic processes.

There may be multiple mechanisms that account for inhibition of elements of both allergen- and ozone-induced inflammation by γ T, especially given that ozone and allergen-induced inflammation likely involve different mechanisms. Ozone is an oxidant gas, which induces epithelial cell injury and production of ozonated lipid mediators, followed by secondary responses of inflammatory cell recruitment. By comparison, allergic inflammation involves activation of mast cells, lymphocytes, epithelial cells and eosinophils. These multiple cell types are sources of various mediators, including eosinophil-derived granular proteins, peroxidases, leukotrienes and interleukins, and mast cell-derived tryptase, histamine and leukotrienes.

Using γ T in models of non-allergic inflammation, we have previously described its ability to inhibit lipoxygenase (LOX) and cyclooxygenase (COX) activities [28, 29]. In the present study we present similar findings of decreased cysLT and a trend for lesser amounts of LTB₄ in BAL fluid. Well known as a neutrophil chemoattractant, a critical role for LTB₄ has recently been described for the recruitment and activation of IL-13-producing T cells [42]. Both IL-13 and cysLTs promote allergic inflammatory responses, including mucin gene expression [36, 43]. These data suggest that the anti-eicosanoid actions of γ T likely account for a significant fraction of the anti-allergic effects we observed in this study.

There are other potential mechanisms by which γ T may minimize inflammatory responses in the airway. One potential common inflammatory mechanism that may play a role in both ozone and allergen-induced inflammation is, production of reactive oxygen species (ROS) and formation of lipid peroxides, as these can be induced by either ozone or allergic stimuli. These oxidant mediators are likely targets for the antioxidant activities of tocopherols such as γ T and α T, both which can directly neutralize superoxide anion, singlet oxygen and lipid peroxy radicals, though γ T is relatively less potent for quenching most ROS than is α T [27].

However, reactive nitrogen species, such as nitric oxide and peroxynitrite, which lead to the nitration of cellular lipids, proteins and nucleotides also cause significant inflammatory changes and injury in tissues during allergy. The protective effects of γ T may be mediated by the neutralization of reactive nitrogen species, such as nitric oxide and peroxynitrite, which lead to the nitration of cellular lipids, proteins and nucleotides. Supporting this idea is the observations that γ T (but not α T) is a highly efficient quencher of nitric oxide and peroxynitrite *in vitro* [26], and that γ T can inhibit protein nitration during systemic inflammation in laboratory rodents [44]. This may be very relevant to allergen-induced inflammation, as nitric oxide production during allergic inflammation, primarily from eosinophils, has been implicated in the nitration and inactivation of key anti-inflammatory and protective enzymes, including catalase, superoxide dismutase, and glutathione transferases [14]. It is reasonable therefore to speculate that γ T could provide protection from nitrosative stress that occurs in allergic airways with eosinophilia. Though we did not assess markers of ROS or RNS in these studies, this is clearly an important focus for subsequent studies of the actions of γ T in asthma.

It has also been reported that γ T can upregulate peroxisome proliferator activated receptor – gamma (PPAR γ), [45, 46] a nuclear transcription factor that regulates many inflammatory genes including those expressed in the asthmatic tissues. Specifically, PPAR γ can modulate eosinophil survival and activation [47], as well as epithelial mucin production [48]. It is possible that the relatively rapid effects of γ T against allergen-induced inflammation could be mediated by an acute activation of PPAR γ to inhibit signaling cascades that lead to airway inflammatory cell recruitment and activation. This is another mechanistic focus for subsequent studies of the effect of γ T on airway inflammation.

In clinical studies, most vitamin E intervention studies directed against ozone-induced respiratory disease have used α T, which is the predominant form of vitamin E in mammalian tissues and for which the recommended dietary allowance (RDA) has been established. Intervention with combination therapy with α T and vitamin C protected from pulmonary function deficits in asthmatic children with decreased intake of dietary antioxidants during high ambient ozone episodes in Mexico City [49], though this effect was most pronounced in children with genetic mutations for glutathione-S-transferase M1, a key enzymatic mediator that opposes pathways of oxidative stress [50]. In a separate study of healthy subjects with limited dietary ascorbate, supplementation with α T, vitamin C and vegetable juice protected them from ozone-induced decrement in pulmonary function but not from airway inflammation [51]. These two studies are in contrast to a recent report by Mudway and colleagues [52], who used supplements with twice the concentrations of α T and vitamin C used above, but failed to inhibit lung function decrements and airway

inflammation after acute 2h ozone exposures. Taken together, these studies suggest that α T is most effective in populations with a decreased intake of dietary antioxidants.

Compared to combination supplements, intervention with large doses of α T alone is much less effective against allergic airway responses. For example, daily supplements of 250mg α T (16 times the RDA of 15mg/d, for 6 weeks) provides no improvement in airway function or bronchodilator use in asthmatics [20]. Doses of 800mg α T /day (53 times RDA) in patients with allergic rhinitis fail to ameliorate symptom severity [22]. Similar results were recently reported in rodents with allergic airways where oral administration of 400 mg/kg/day α T for 10 days failed to reduce allergen-induced pulmonary lesions or airway hyperreactivity [21]. Using a very similar allergen sensitization and challenge protocol, we observed inhibition of ozone and allergen-induced allergic inflammation with only 4 days of treating with a much smaller dose of γ T. This comparison is reminiscent of our findings in a non-immunogenic inflammation in rats, where γ T was superior to α T in limiting inflammatory cell recruitment and cytokine production [29]. The role of γ T in ozone-induced airway responses has not been extensively examined in either laboratory animals or humans. However, acute ozone exposure to BALB/c mice preferentially increased γ T, ascorbate and uric acid in BAL fluid compared to α T [53], which is similar to the trend we detect for BALF γ T from ozone-exposed rats in the present study. These results in laboratory rodents suggest that the γ -isoform of vitamin E may play a relatively more important role in regulating pulmonary inflammation than α T

Taken together, we have demonstrated broad and novel anti-inflammatory effects of γ T after exposure to OVA allergen and/or ozone, two disparate stimuli. We furthermore described therapeutic effects with a relatively acute dosing regimen, and suggest that γ T does not require prophylactic use or repeated doses to produce efficacy. On the basis of these observations in laboratory animals, we are presently developing a rodent study to examine the effect of γ T on airway inflammation induced only by allergen as well as a series of phase I studies to test the safety and efficacy of γ T in allergic people coexposed to allergens and environmental pollutants.

Acknowledgements

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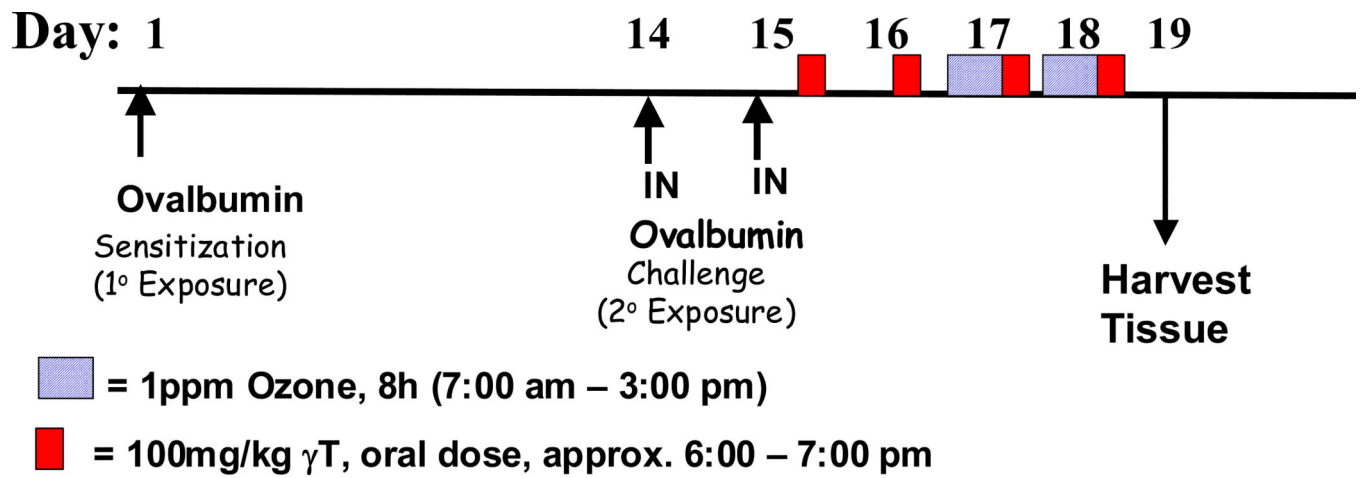


Figure 1. Experimental Protocol

Ovalbumin-sensitized Brown Norway rats were challenged with intranasal ovalbumin (Days 14, 15) and then exposed to 0 or 1.0 ppm ozone (Days 17 & 18). Animals were given γ T of vehicle beginning after challenge daily for the duration of the study (Days 15-18).

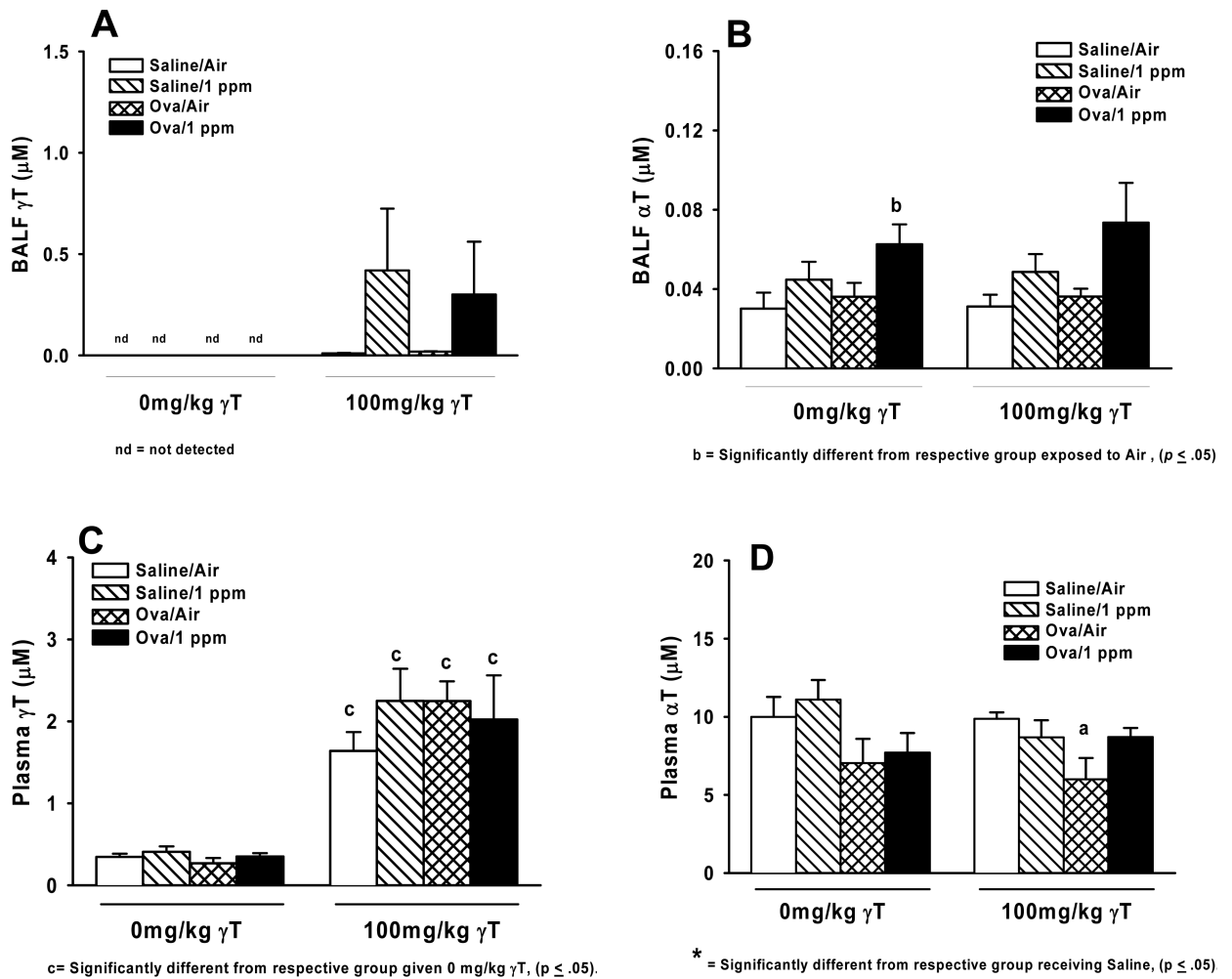


Fig 2. Tissue tocopherol content

Normal and allergic Brown Norway rats were exposed to 0 or 1.0 ppm ozone and treated orally with 0 or 100 mg/kg γ T as described previously. Plasma and bronchoalveolar lavage fluid was collected and analyzed for content of α - and γ -tocopherol by HPLC as described in Materials and Methods. Data are expressed as mean \pm SEM. a = significantly different from respective group challenged with intranasal saline; b = significantly different from respective group exposed to 0 ppm ozone; c = significantly different from respective group treated with 0 mg/kg γ T. Criteria for significance, $p < 0.05$.

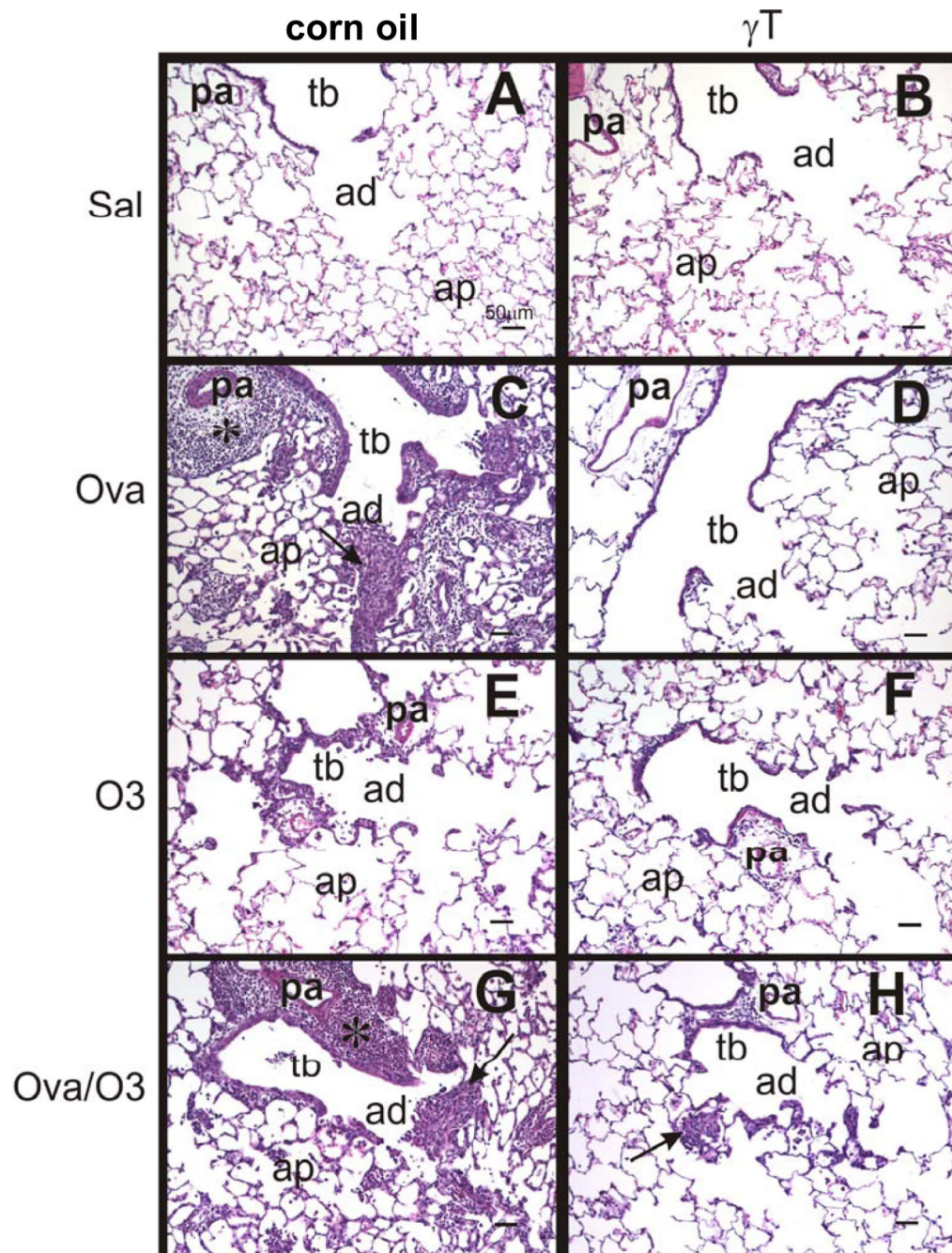


Figure 3. Photomicrographs of the epithelium of conducting airways in lungs of rats challenged with saline (Sal) and treated with either corn oil (A) or γ T (B); challenged with Ova and treated with corn oil (C) or γ T (D); exposed to ozone (O3) and treated with corn oil (E) or γ T (F); or challenged with Ova and exposed to ozone (Ova/O3), and then treated with corn oil (G) or γ T (H). Tissues were stained with H&E. pa = pulmonary artery, tb = terminal bronchiole, ad = alveolar duct, ap = alveolar parenchyma.

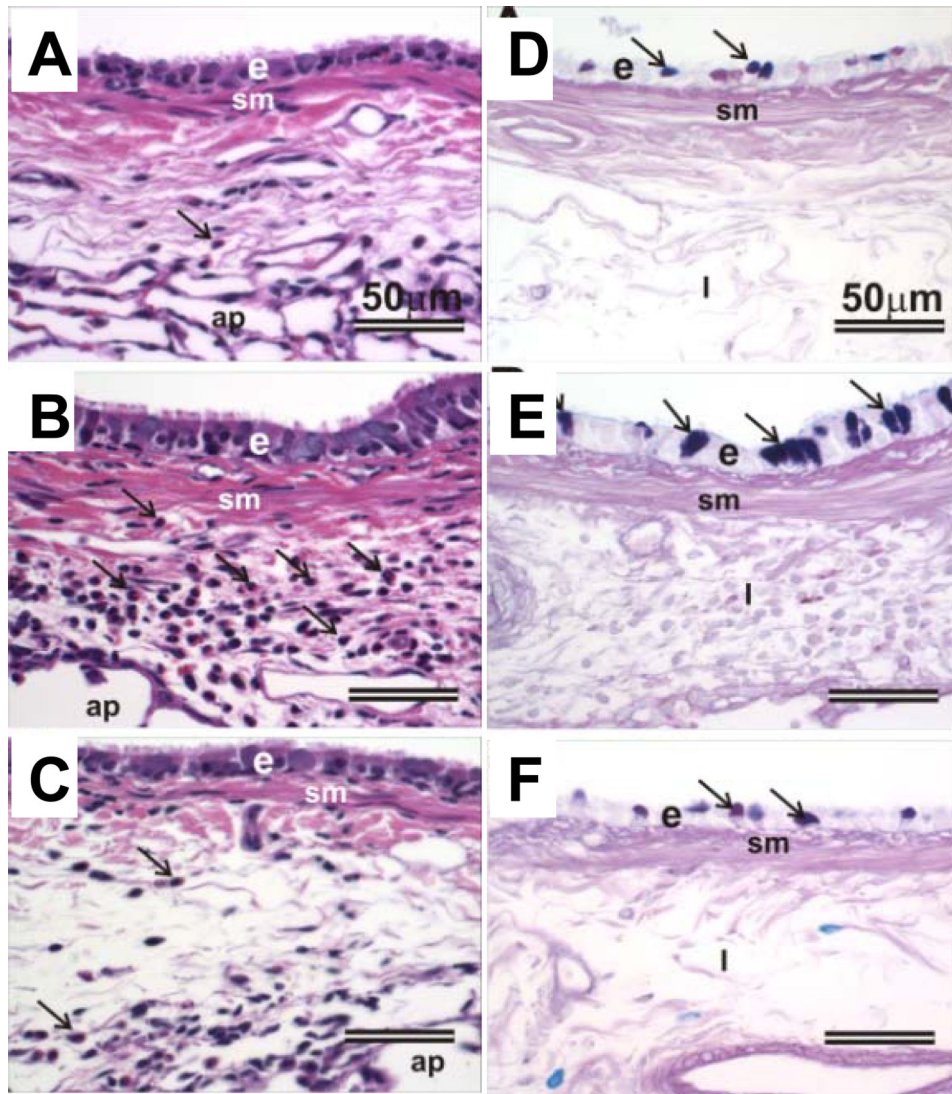


Figure 4. Photomicrographs of the respiratory airway epithelium lining the axial airway in lungs of rats (A,D) challenged with saline and treated with corn oil respectively, (B,E) challenged with OVA, exposed to ozone and treated with corn oil, or (C,F) challenged with OVA, exposed to ozone and treated with γ T. Tissues were stained with H&E (A,B,C) or AB/PAS (D,E,F) to identify intraepithelial mucosubstances (identified by arrows). e = epithelium; sm = smooth muscle; i = interstitium; arrowheads in H&E = inflammatory cells.

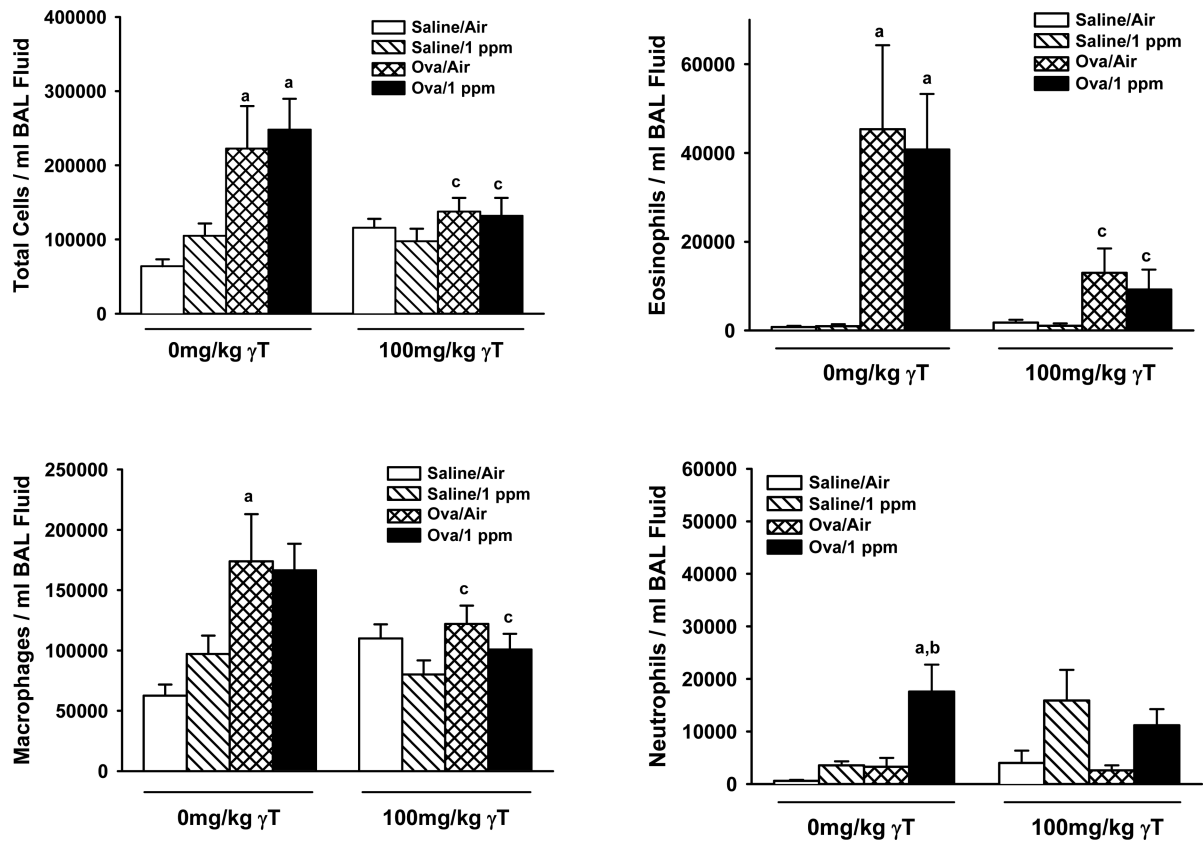


Figure 5. Effect of γ T on Inflammatory Cells Recovered in Bronchoalveolar Lavage Fluid

Normal and allergic Brown Norway rats were exposed to 0 or 1.0 ppm ozone and treated orally with 0 or 100 mg/kg γ T as described previously. Bronchoalveolar lavage fluid was collected and cell enumerated as described in Materials and Methods. Data are expressed as mean \pm SEM. a = significantly different from respective group challenged with intranasal saline; b = significantly different from respective group exposed to 0 ppm ozone; c = significantly different from respective group treated with 0 mg/kg γ T. Criteria for significance, $p < 0.05$.

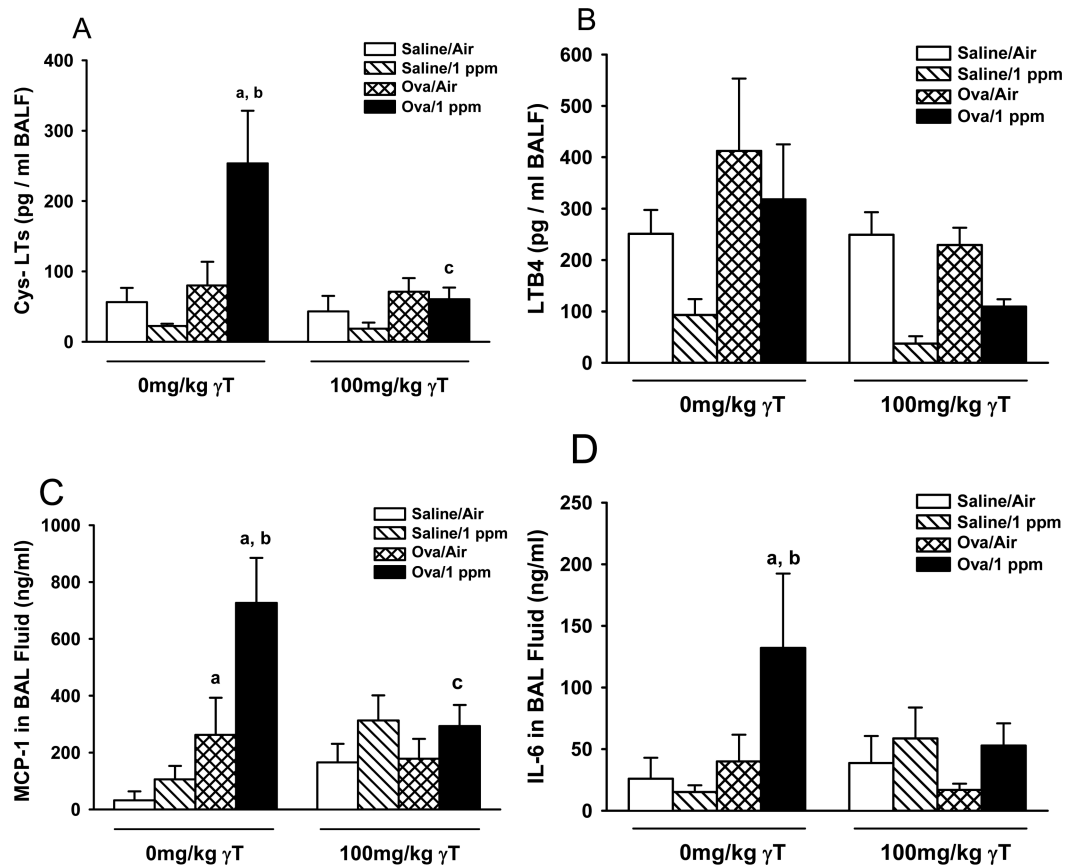


Figure 6. Soluble Inflammatory Mediators in BALF

Normal and allergic Brown Norway rats were exposed to 0 or 1.0 ppm ozone and treated orally with 0 or 100 mg/kg γ T as described previously. Bronchoalveolar lavage fluid was collected and analyzed for cytokine and leukotrienes as described in Materials and Methods. Data are expressed as mean \pm SEM. a = significantly different from respective group challenged with intranasal saline; b = significantly different from respective group exposed to 0 ppm ozone; c = significantly different from respective group treated with 0 mg/kg γ T. Criteria for significance, $p < 0.05$

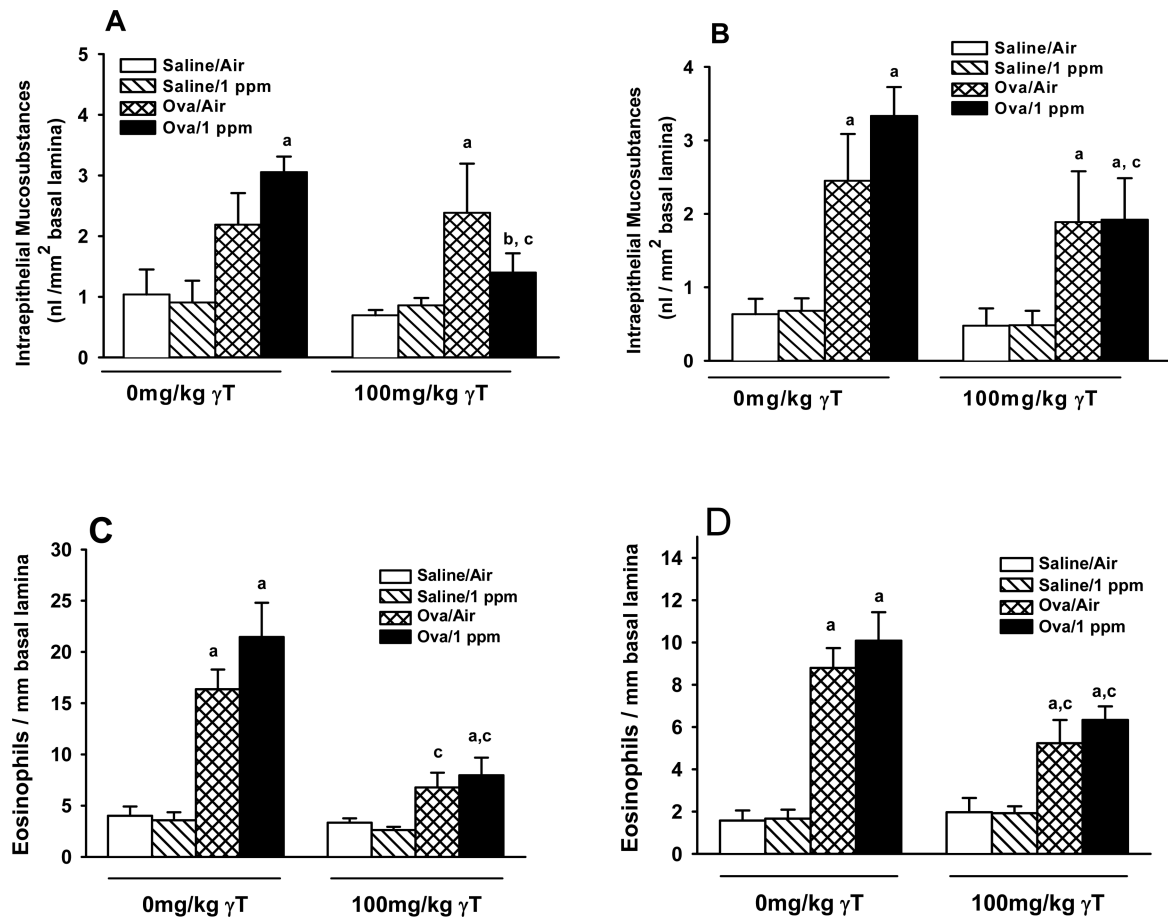


Figure 7. Effect of γ T on Ozone-Induced Enhancement of Allergic Mucous Cell Metaplasia and Eosinophilic Inflammation in Pulmonary Axial Airways

Normal and allergic Brown Norway rats were exposed to 0 or 1.0 ppm ozone and treated orally with 0 or 100 mg/kg γ T as described previously. Lungs sections from proximal (A) and distal (B) axial airways were stained with AB/PAS and intraepithelial mucosubstances were morphometrically analyzed as described in Materials and Methods. Lungs stained with May Grunwald were used to quantify eosinophils in proximal (C) and distal (D) axial airways. Data are expressed as mean \pm SEM. a = significantly different from respective group challenged with intranasal saline; b = significantly different from respective group exposed to 0 ppm ozone; c = significantly different from respective group treated with 0 mg/kg γ T. Criteria for significance, $p < 0.05$.

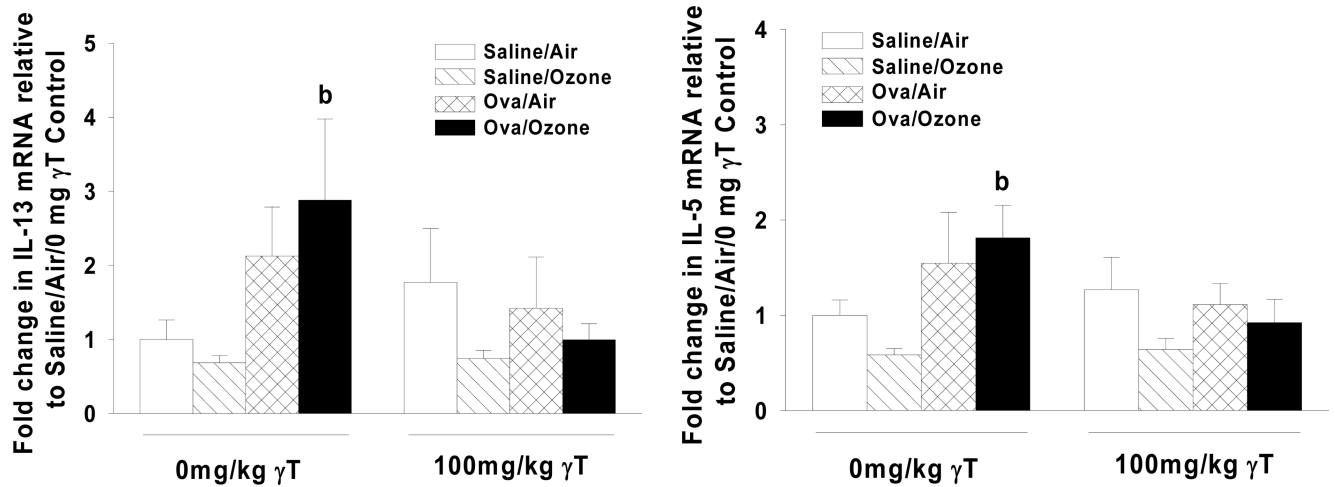


Figure 8. Effect of γ T on Inflammatory Gene Expression in Lung Tissue

Normal and allergic Brown Norway rats were exposed to 0 or 1.0 ppm ozone and treated orally with 0 or 100 mg/kg γ T as described previously. RNA was isolated from the cranial lung lobe and analyzed by RT-PCR as described in Materials and Methods. Data are expressed as mean \pm SEM. a = significantly different from respective group challenged with intranasal saline; b = significantly different from respective group exposed to 0 ppm ozone; c = significantly different from respective group treated with 0 mg/kg γ T. Criteria for significance, $p < 0.05$.