

## ORIGINAL PAPER

# $\gamma$ -Tocopherol prevents airway eosinophilia and mucous cell hyperplasia in experimentally induced allergic rhinitis and asthma

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## Clinical and Experimental Allergy

### Summary

**Background** Traditional therapies for asthma and allergic rhinitis (AR) such as corticosteroids and antihistamines are not without limitations and side effects. The use of complementary and alternative approaches to treat allergic airways disease, including the use of herbal and dietary supplements, is increasing but their efficacy and safety are relatively understudied. Previously, we have demonstrated that  $\gamma$ -tocopherol ( $\gamma$ T), the primary form of dietary vitamin E, is more effective than  $\alpha$ -tocopherol, the primary form found in supplements and tissue, in reducing systemic inflammation induced by non-immunogenic stimuli.

**Objective** We used allergic Brown Norway rats to test the hypothesis that a dietary supplement with  $\gamma$ T would protect from adverse nasal and pulmonary responses to airway allergen provocation.

**Methods** Ovalbumin (OVA)-sensitized Brown Norway rats were treated orally with  $\gamma$ T before intranasal provocation with OVA. Twenty-four hours after two challenges, histopathological changes in the nose, sinus and pulmonary airways were compared with gene expression and cytokine production in bronchoalveolar lavage fluid and plasma.

**Results** We found that acute dosing for 4 days with  $\gamma$ T was sufficient to provide broad protection from inflammatory cell recruitment and epithelial cell alterations induced by allergen challenge. Eosinophil infiltration into airspaces and tissues of the lung, nose, sinus and nasolacrimal duct was blocked in allergic rats treated with  $\gamma$ T. Pulmonary production of soluble mediators PGE<sub>2</sub>, LTB<sub>4</sub> and cysteinyl leukotrienes, and nasal expression of IL-4, -5, -13 and IFN- $\gamma$  were also inhibited by  $\gamma$ T. Mucous cell metaplasia, the increase in the number of goblet cells and amounts of intraepithelial mucus storage, was induced by allergen in both pulmonary and nasal airways and decreased by treatment with  $\gamma$ T.

**Conclusions** Acute treatment with  $\gamma$ T inhibits important inflammatory pathways that underlie the pathogenesis of both AR and asthma. Supplementation with  $\gamma$ T may be a novel complementary therapy for allergic airways disease.

**Keywords** complementary medicine, eosinophil, IFN- $\gamma$ , leukotrienes, mucous cell metaplasia, tocopherol

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### Introduction

Clinical and animal studies indicate that allergic airways disease is mediated in part by an imbalance of oxidant and antioxidant species that is associated with symptom severity. Consistent with this idea are observations that decreases in antioxidant vitamins E and C, and increases in oxidized glutathione are common in asthmatic airways [1–3]. Host antioxidant defences in the lung and plasma of

asthmatics are also depressed with lower activities of superoxide dismutase, catalase, and glutathione peroxidase [4–6].

$\alpha$ -Tocopherol ( $\alpha$ T), the major form of vitamin E in mammalian tissues, has been postulated as a therapy for airway allergic inflammation due to its ability to neutralize lipid radicals and inhibit cyclooxygenase (COX) and leukotriene synthesis [7]. In studies by Romieu and colleagues [8–10], combination therapy with  $\alpha$ T and

ascorbate was found to protect children with allergic asthma against ozone-induced increases in asthma symptoms and nasal inflammation. However, in both human and animal studies, intervention with  $\alpha$ T supplements alone failed to improve constitutive allergic airway symptoms appreciably [11–13]. These mixed results have dampened the enthusiasm for use of  $\alpha$ T supplements in the prevention of allergic disease.

Nonetheless, deficits in the antioxidant repertoire in allergic airways are almost certainly linked to a lower dietary intake of natural sources of antioxidants, such as from fruits and vegetables. Recent epidemiological studies have shown that children born to mothers who consume decreased amounts of vitamin E and other antioxidants have a significantly increased risk of developing asthma [14, 15]. Furthermore, case studies have reported that the intake of vitamins C, E and carotene by asthmatics was inversely correlated with the severity of symptoms [16–18], and overall, asthmatics tend to have lower amounts of antioxidant nutrients in their diets, especially of vitamins E and C [19].

Others and we have previously shown that  $\gamma$ -tocopherol ( $\gamma$ T), the major form of vitamin E in US diets, is superior to  $\alpha$ T in attenuating inflammation and associated tissue injury.  $\gamma$ T appears to be more powerful than  $\alpha$ T in trapping electrophiles such as reactive nitrogen species, which play important roles in mediating allergic inflammation [20, 21]. Compared with  $\alpha$ T,  $\gamma$ T and its primary metabolite, 2,7,8-trimethyl-2-( $\beta$ -carboxy-ethyl)-6-hydroxychroman, ( $\gamma$ CEHC), more potently inhibited COX-catalysed pro-inflammatory prostaglandins in cultured cells [22] and in a rat model of inflammation [23]. Besides pro-inflammatory eicosanoids, we have shown that  $\gamma$ T is superior to  $\alpha$ T for inhibition of TNF- $\alpha$ , nitrate/nitrite and inflammation-caused damage. We therefore hypothesize that the  $\gamma$ T form of vitamin E might be, at least in part, responsible for the protective effects seen in dietary case studies of asthmatics.

In the present study, we used a rodent model of allergic airways disease to test the hypothesis that dietary supplementation with  $\gamma$ T would protect from allergen-induced airway inflammatory responses. We demonstrate herein, using histological, morphological, biochemical and molecular approaches, that  $\gamma$ T inhibits inflammatory and epithelial cell responses in both the upper and the lower airways. Specifically, we document attenuation by  $\gamma$ T of allergen-induced inflammatory cell recruitment, cytokine gene expression and overproduction of mucus that characterizes allergic rhinoconjunctivitis and asthma.

## Methods

### Animals

Male Brown Norway rats (Harlan, Indianapolis, IN, USA), 10–12 weeks of age, 230–260 g, were used in all studies.

Animals were free of pathogens, and used in accordance with guidelines set forth by the All-University Committee on Animal Use and Care at Michigan State University. Rats had free access to water and food (Harlan Teklad 22/5 Rodent Diet 8640; Harlan Teklad, Madison, WI, USA) throughout the study with a daily intake of approximately 1.7 mg  $\alpha$ T.

### Study 1: Dose-ranging protocol for $\gamma$ -tocopherol

Twenty-four animals (eight groups;  $n=3$ /group) were administered by oral gavage 0, 33 or 100 mg/kg (body weight) of  $\gamma$ T (highly purified r,r,r isomer; Yasoo Health Inc., Johnson City, TN, USA) prepared in tocopherol-stripped corn oil (CO) (Dyets Inc., Bethlehem, PA, USA) for 2, 3 or 4 consecutive days. CO and  $\gamma$ T were prepared and flushed with nitrogen and stored at 4 °C. Twenty-four hours after the last supplement with  $\gamma$ T, animals were killed, lung tissues were removed and blotted lightly on gauze and then snap frozen in liquid nitrogen.

### Study 2: Supplementation during allergic airway exacerbation

Twenty-eight animals (four groups;  $n=7$ /group) were used to determine the efficacy of  $\gamma$ T on allergic airway responses. Rats were sensitized to chicken albumin [ovalbumin (OVA); Sigma Chemical Co., St Louis, MO, USA] by an intraperitoneal injection with 2.5 mL sterile saline of a solution containing 1 mg OVA prepared with 10 mg alum as an adjuvant. Two weeks later, rats were challenged with OVA or saline for 2 consecutive days (Days 1 and 2) under light anaesthesia (4% halothane in oxygen) by intranasal instillation (IN) with 150  $\mu$ L of 0 or 0.5% OVA into each nasal passage (300  $\mu$ L total volume).

Based on results from the pharmacokinetic study, we used 100 mg/kg of  $\gamma$ T to assess its efficacy in allergic airways. Thus, 2 days before IN challenge with OVA (i.e. Day –2), rats were administered by oral gavage 0 or 100 mg/kg body weight of  $\gamma$ T in CO. Oral administration of  $\gamma$ T was given daily at 6:00 hours for four consecutive days (Days –2, –1, 1 and 2). This 4-day dosing protocol resulted in 2 days of  $\gamma$ T pre-treatment (Days –2, –1), and 2 days of treatment on days of allergen challenge (Days 1 and 2). This design consisted of four groups of seven animals: non-allergic, CO; non-allergic,  $\gamma$ T; allergic, CO; and allergic,  $\gamma$ T. Animals were euthanized and tissues were collected on Day 3, approximately 24 h after the second of two allergen challenges.

### Necropsy, lavage collection, and tissue preparation

Animals were anaesthetized 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 haemostatic clamp, and 4 mL of sterile saline was infused 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 placed in RNAlater (Qiagen Inc., Valencia, CA, USA). The right cranial lobe was also excised and placed in RNAlater. Samples were kept at  $-80^{\circ}\text{C}$  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  $\text{H}_2\text{O}$ ) with neutral-buffered formalin for 2 h, while immersed in a large volume of fixative. Heads were removed and placed in fixative. Twenty-four hours later, two sections were excised from the left-lung lobe at the level of the fifth and 11th airway generation, to sample proximal and distal airways, respectively [26]. Heads were decalcified in 13% formic acid for 5 days. Two nasal sections were taken from the anterior nasal cavity by making perpendicular cuts to the hard palate at the level (1) immediately posterior to the upper incisors and (2) at the incisive papilla. Tissue blocks were then embedded in paraffin, and 5–6  $\mu\text{m}$  thick sections were cut from the anterior surface. Lung and nasal sections were stained with haematoxylin and eosin for routine histology, with Alcian blue (pH 2.5)/periodic acid-Schiff to detect intraepithelial mucosubstances (IM), or with May Grunwald stain to detect eosinophils. Site-specific morphometry of IM and eosinophils was conducted in the epithelium and underlying mucosa in the proximal and distal axial pulmonary conducting airways, proximal and distal nasal septum, maxillary sinus and the nasolacrimal duct.

#### *Bronchoalveolar lavage fluid/plasma analysis*

Total leucocytes in BALF were enumerated with a haemocytometer, and fractions of eosinophils, neutrophils, macrophages and lymphocytes were determined in a cyto-spin sample stained with Diff-Quick (Dade Behring, Newark, DE, USA).

**Cytokine and leukotriene content.** For leukotriene analysis, cell-free BALF was first purified in 2 mL methanol and 5 mL hexane to precipitate proteins and lipid, respectively. The methanol layer containing leukotrienes was dried under  $\text{N}_2$  stream. The amount of LTC<sub>4</sub> and total cysteinyl-leukotrienes (LTC<sub>4</sub>, LTD<sub>4</sub> and LTB<sub>4</sub>) were measured using an EIA assay from Cayman Chemicals (Ann Arbor, MI, USA). The cytokine content in BALF was determined

using a custom bead-based multiplex assay capable of detecting up to 15 different rat protein targets. Assays were conducted in 96-well MultiScreen plates (Millipore, Billerica, MA, USA) by the same principle of standard ELISA. Bound protein:antibody conjugates on beads are detected using a Streptavidin-PE system and analysed using the Luminex<sup>100</sup> total system (Luminex Corp, Austin, TX, USA).

**Tocopherols.** Plasma, BALF and lung homogenate content of  $\gamma\text{T}$  and  $\alpha\text{T}$  were extracted using a mixture of methanol/hexane (2:5, v/v) in the presence of 0.8 mM butylated hydroxytoluene (BHT). After a brief centrifugation at  $4^{\circ}\text{C}$ , the top hexane layer was dried under  $\text{N}_2$  and the residue was resuspended in ethanol. Tocopherols were separated on a  $150 \times 4.6$  mm, 5  $\mu\text{m}$  Supelcosil<sup>TM</sup> LC-18-DB column (Supelco, Bellefonte, PA, USA) and eluted with 95:5 (v/v) methanol/0.1 M 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. Tocopherol concentrations in the experimental samples were estimated by comparison with standard curves of  $\alpha\text{T}$  and  $\gamma\text{T}$ .

#### *Real-time reverse transcription-polymerase chain reaction*

Total RNA was extracted using the Rneasy Mini Kit according to the manufacturers instruction's (Qiagen). The evaluation of the relative expression levels of IL-4, -5, -13 and IFN- $\gamma$  mRNA was performed by two-step RT-PCR according to the manufacturer's 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, Foster City, CA, USA). Relative gene expression was normalized to 18S. Data are expressed as fold-increase in RNA expression compared with control animals, which are set at a value of one.

#### *Morphometry of stored intraepithelial mucosubstances*

To estimate the amount of the IM in respiratory epithelium lining pulmonary axial airways, the volume density of IM was quantified using computerized image analysis and standard morphometric techniques. The area of IM was calculated from the automatically circumscribed perimeter of stained material using the public domain NIH Image programme (written by Wayne Rasband, US 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 of stored mucosubstances per unit of surface area of epithelial basal lamina was estimated using the methods described previously [27]. The volume density of IM is expressed as nanolitres per mm square 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, was determined by counting May–Grunwald-positive-stained cells with a distinct eosinophil morphology. The length of the basal lamina was calculated as described above, and the data are expressed as the number of eosinophils divided by the length of the underlying basal lamina.

#### Statistical analysis

Data are expressed as mean  $\pm$  SE. Grubb's outlier test was used to determine statistical outliers. Data were analysed using a completely randomized analysis of variance (SigmaStat, Jandel, Scientific, CA, USA). Multiple comparisons were made by the Student–Newman–Keuls *post hoc* test. The criterion for significance was taken to be  $P \leq 0.05$ .

### Results

#### Tocopherol content in lung tissue

Supplementation of rats with 33 mg/kg  $\gamma$ T for 2, 3 or 4 days increased the lung content of  $\gamma$ T threefold compared with corn-oil controls, and was independent of the number of days of supplementation (Fig. 1). Administration of 100 mg/kg  $\gamma$ T caused sixfold increases in pulmonary  $\gamma$ T concentrations by comparison, and was also independent of the number of  $\gamma$ T administrations.

#### Tocopherol content in plasma and bronchoalveolar lavage fluid

Treatment with 100 mg/kg  $\gamma$ T caused the plasma levels of  $\gamma$ T to increase by three to four-fold, irrespective of allergen (OVA) challenge (Table 1). Plasma  $\alpha$ T levels were unaffected by  $\gamma$ T treatment. Trends for decreased  $\alpha$ T in plasma from non-allergic ( $P = 0.096$ ) and allergic ( $P = 0.102$ ) rats were not different after  $\gamma$ T treatment. In BALF, the  $\gamma$ T content was minimal or undetectable from CO vehicle-treated rats. Supplementation with  $\gamma$ T produced measurable levels in all BAL samples, but there were no differences in allergic vs. non-allergic rats given  $\gamma$ T. Changes in lavage  $\alpha$ T levels were not statistically significant.

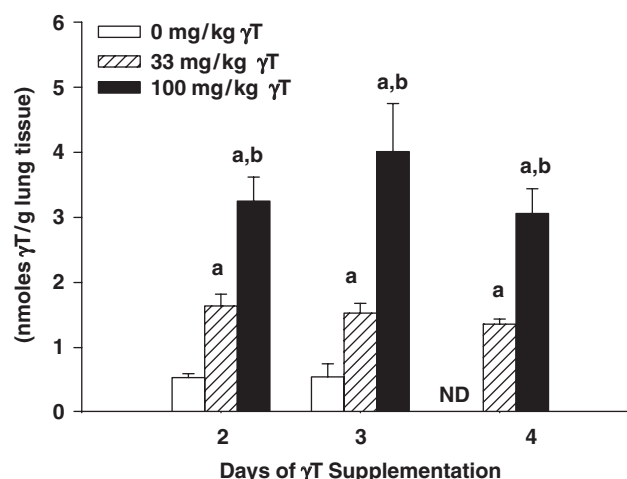


Fig. 1. Effect of  $\gamma$ -tocopherol ( $\gamma$ T) supplementation on lung  $\gamma$ T content. Naïve animals (non-sensitized) were supplemented with 0, 33 or 100 mg/kg  $\gamma$ T, p.o. for 2, 3 or 4 consecutive days. Twenty-four hours after the last treatment, the lungs were collected and analysed for tocopherol content as described in Methods. <sup>a</sup>significantly different from 0 mg/kg  $\gamma$ T; <sup>b</sup>significantly different from 33 mg/kg  $\gamma$ T; <sup>nd</sup>not done:  $\gamma$ T supplemented groups in 4 day group were compared with 0 mg/kg controls from days 1 and 2.  $n = 3$ /group;  $P < 0.05$ .

#### Bronchoalveolar lavage

**Cellularity.** Challenge with OVA induced an increase of total cells, eosinophils, neutrophils and lymphocytes in BALF, while increases in macrophages were not statistically significant (Table 2). Treatment with  $\gamma$ T attenuated OVA-induced increases in BALF eosinophils (74% decrease) and lymphocytes (100% decrease), whereas decreases in total cells and neutrophils were not statistically significant (48% and 38% decreases, respectively).

**Leukotrienes and cytokines.** Treatment with  $\gamma$ T had no significant effect on BALF cysteinyl leukotrienes (cys-LTs), LTB<sub>4</sub> or PGE<sub>2</sub> in non-allergic rats (Table 3). OVA challenge caused increased production and accumulation of cys-LTs in BALF, but had no effect on LTB<sub>4</sub> and PGE<sub>2</sub>. In allergic rats given  $\gamma$ T, cys-LTs were not statistically different from their respective, non-allergic control rats ( $P = 0.06$ ); however, neither were they different from allergic rats treated with CO vehicle. Treatment with  $\gamma$ T caused a reduction of LTB<sub>4</sub> and PGE<sub>2</sub> in allergic rats, with a trend towards decreased PGE<sub>2</sub> in non-allergic animals ( $P = 0.10$ ).

#### Histopathology

**Pulmonary pathology.** No exposure-related lesions were found in the lungs of control rats that were intranasally instilled with only saline and gavaged with CO alone or with  $\gamma$ T in CO (Fig. 2). In contrast, rats that were intranasally challenged with OVA and gavaged with CO alone had

**Table 1.** Effect of γ-tocopherol (γT) on bronchoalveolar lavage and plasma tocopherols (μM)

| Experimental group     | Plasma γT     | Plasma αT    | Lavage γT       | Lavage αT     |
|------------------------|---------------|--------------|-----------------|---------------|
| Saline/corn oil        | 0.532 ± 0.063 | 10.93 ± 0.82 | 0 ± 0           | 0.023 ± 0.011 |
| Saline/γ-tocopherol    | 1.49 ± 0.35 b | 9.04 ± 1.06  | 1.35 ± 0.63 b   | 0.079 ± 0.021 |
| Ovalbumin/corn oil     | 0.49 ± 0.076  | 8.99 ± 0.51  | 0.002 ± 0.002   | 0.070 ± 0.058 |
| Ovalbumin/γ-tocopherol | 2.00 ± 0.43 b | 6.95 ± 0.69  | 0.045 ± 0.013 b | 0.235 ± 0.117 |

b, significantly different from respective group given corn oil  $P < 0.05$ .

**Table 2.** Effect of γ-tocopherol treatment on bronchoalveolar lavage cellularity (cells/mL)

| Experimental group     | Total cells         | Monocytes        | Eosinophils         | Neutrophils         | Lymphocytes     |
|------------------------|---------------------|------------------|---------------------|---------------------|-----------------|
| Saline/corn oil        | 80 416 ± 11 354     | 73 624 ± 12 387  | 2278 ± 902          | 1970 ± 1090         | 2561 ± 608      |
| Saline/γ-tocopherol    | 100 417 ± 9967      | 84 326 ± 13 348  | 1617 ± 384          | 12 064 ± 5085       | 2430 ± 486      |
| Ovalbumin/corn oil     | 663 333 ± 176 490 a | 109 904 ± 21 246 | 148 597 ± 50 287 a  | 392 955 ± 134 004 a | 12 088 ± 3086 a |
| Ovalbumin/γ-tocopherol | 425 833 ± 133 732 a | 103 880 ± 17 893 | 40 812 ± 12 647 a,b | 245 480 ± 101 128 a | 2470 ± 777      |

a, significantly different from respective group challenged with saline,  $P < 0.05$ .

b, significantly different from respective group given corn oil,  $P < 0.05$ .

**Table 3.** Effect of γ-tocopherol on bronchoalveolar lavage eicosanoids (ng/mL)

| Experimental group     | LTC <sub>4</sub> | LTB <sub>4</sub> | PGE <sub>2</sub> |
|------------------------|------------------|------------------|------------------|
| Saline/corn oil        | 0.089 ± 0.035    | 0.439 ± 0.136    | 0.223 ± 0.023    |
| Saline/γ-tocopherol    | 0.051 ± 0.013    | 0.252 ± 0.072    | 0.133 ± 0.021    |
| Ovalbumin/corn oil     | 0.271 ± 0.080 a  | 0.394 ± 0.100    | 0.268 ± 0.061    |
| Ovalbumin/γ-tocopherol | 0.220 ± 0.064    | 0.107 ± 0.009 b  | 0.087 ± 0.005 b  |

a, significantly different from respective group challenged with saline,  $P < 0.05$ .

b, significantly different from respective group given corn oil,  $P < 0.05$ .

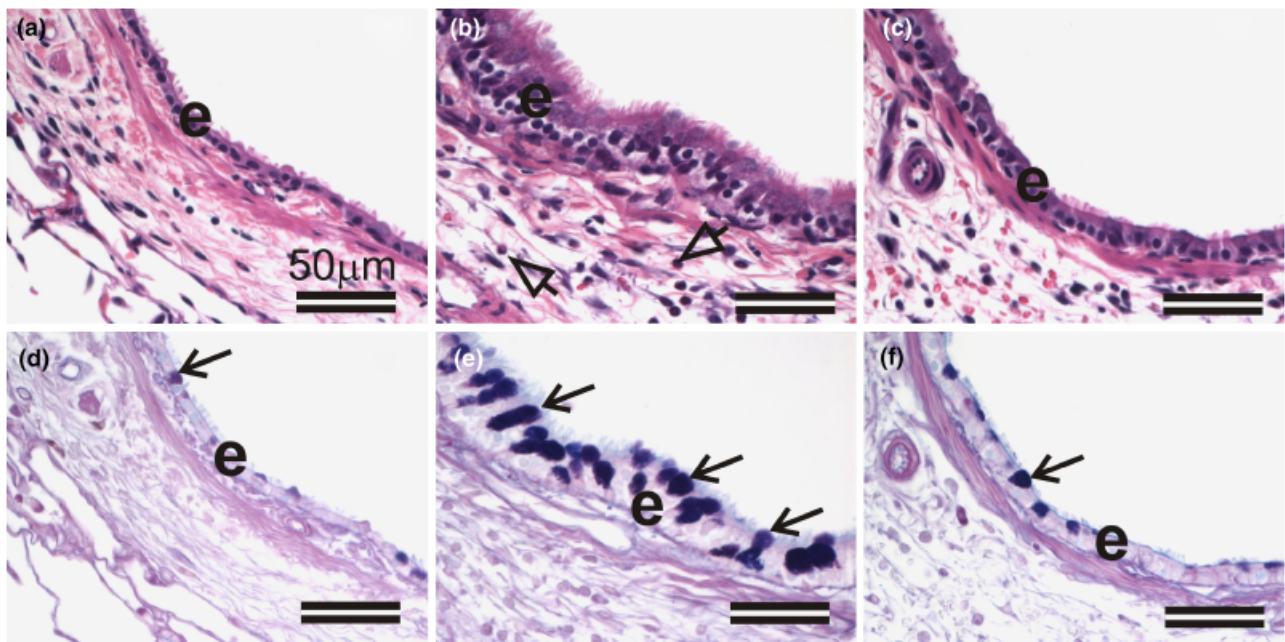


**Fig. 2.** Effect of γ-tocopherol (γT) on histopathological responses in pulmonary airways. Photomicrographs of the respiratory airway epithelium lining the axial airway (generation 11) in the lungs of rats intranasally instilled and gavaged with (a, d), saline and corn oil (CO) respectively, (b, e) ovalbumin (OVA) and CO, or (c, f) OVA and γT. Tissues were stained with haematoxylin and eosin (a–c) or AB/PAS (d–f) to identify intraepithelial mucosubstances (identified by arrows). e, epithelium; open arrowheads, inflammatory cells.

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 were more marked than those in the distal lung lobe section, indicating a proximal–distal decrease in the lobar lesion severity and distribution.

OVA-challenged rats also had a conspicuous hypertrophy (tall columnar epithelial cells) and mucous cell hyperplasia/metaplasia (MCH) with increased amounts of AB/PAS-stained IM in the respiratory epithelium lining the large-diameter intrapulmonary airways, including the proximal axial airway (Figs 2b and e), and to a much lesser extent in the distal axial airway and the small-diameter preterminal and terminal bronchioles. In contrast, saline-instilled rats and gavaged with CO alone or  $\gamma$ T in CO had significantly fewer mucous cells and IM in a much thinner, low columnar, respiratory epithelium lining large-diameter airways (Figs 2a and d). 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. The terminal bronchioles of saline-instilled rats contained no mucous cells.

Treatment with  $\gamma$ T markedly attenuated OVA-induced epithelial and inflammatory lesions in both the intrapulmonary proximal and distal conducting airways and in the alveolar parenchyma (Figs 2c and f). There was only

minimal to mild airway epithelial thickening with mucous cell metaplasia (MCM) and peribronchial inflammatory cell influx in both the small- and large-diameter bronchioles in the OVA-challenged rats treated with  $\gamma$ T. In contrast,  $\gamma$ T treatment caused only a modest attenuation of the OVA-induced lesions in the alveolar parenchyma (not shown).

**Nasal pathology.** Control rats treated with CO alone, or  $\gamma$ T in CO, and intranasally instilled with saline had no exposure-related nasal lesions (Figs 3a and d). Rats intranasally challenged with OVA had a mild to moderate allergic rhinitis (AR). This nasal response was characterized by a mixed inflammatory cell infiltrate mainly of eosinophils and lymphocytes, and lesser numbers of neutrophils and plasma cells, in the nasal mucosa lining most of the intranasal airways in both the proximal and the distal nasal passages.

The conspicuous eosinophilic and lymphocytic inflammatory cell response was, however, restricted to (1) the nasal mucosa containing ciliated, pseudostratified respiratory epithelium lining the proximal and distal nasal septum (Figs 3b and e), distal lateral meatus and maxillary sinus, and (2) mucosa containing non-ciliated cuboidal epithelium (nasal transitional epithelium) lining the proximal lateral meatus. A similar inflammatory cell infiltrate was also evident in the tissues surrounding the nasolacrimal duct. Interestingly, no OVA-induced inflammatory cell response was histologically evident in the nasal mucosa containing the olfactory epithelium.

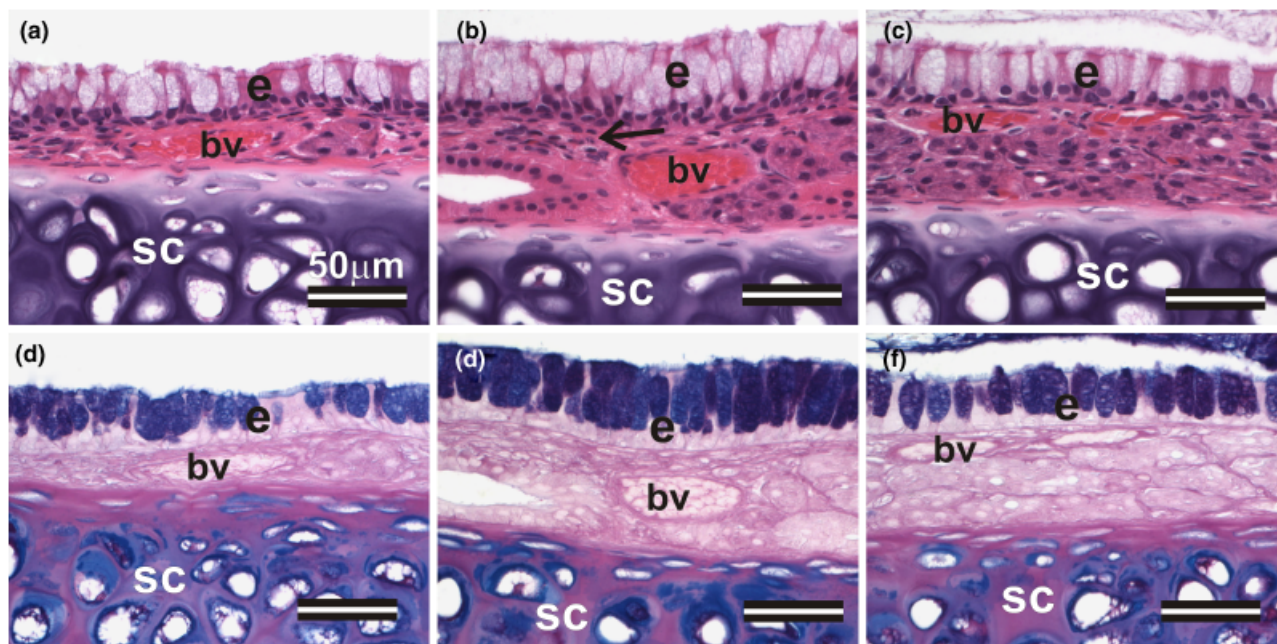


Fig. 3. Effect of  $\gamma$ -tocopherol ( $\gamma$ T) on histopathological responses in nasal airways. Photomicrographs of the respiratory airway epithelium lining the midseptum in the proximal nasal airways of rats intranasally instilled and gavaged with (a, d) saline and corn oil (CO) respectively, (b, e) ovalbumin (OVA) and CO or (c, f) OVA and  $\gamma$ T. Tissues were stained with haematoxylin and eosin (a–c) or AB/PAS (d–f) to identify intraepithelial mucosubstances (dark blue). e, epithelium; arrow, inflammatory cells; bv, blood vessel; SC, septal cartilage.

In addition to the OVA-induced inflammatory response, the nasal respiratory epithelium lining of all these regions was conspicuously thickened with MCH (Fig. 3b). The amount of AB/PAS-stained mucosubstances in the mucous cells of this nasal epithelium was markedly increased compared with that of the saline-instilled control rats (Figs 3d and e).

Treatment with  $\gamma$ T markedly attenuated the OVA-induced epithelial and inflammatory lesions in both the proximal and the distal nasal passages (Figs 3c and f). There was only minimal to mild airway epithelial thickening with MCH in these  $\gamma$ T-treated rats.

### Nasal and pulmonary morphometry

**Mucous cell metaplasia.** Two consecutive days of OVA challenge-induced MCM as indicated by significant increases in IM in conducting proximal airways of lungs (Fig. 4a). Treatment with  $\gamma$ T attenuated these allergen-induced responses. In the nose, IM increased 100% in proximal nasal septum, which was significantly decreased in rats given  $\gamma$ T (Fig. 4b). Allergen-induced MCM was also blocked by  $\gamma$ T in the distal pulmonary airways and in the distal nasal septum (data not shown).

**Eosinophilic inflammation.** Concomitant with MCM, OVA challenge induced a three to sixfold increase in the

number of eosinophils in the submucosa of conducting pulmonary airways and in the nasal septum (Figs 5a and b). Significant recruitment of eosinophils was also detected in the duct leading to the maxillary sinus (Fig. 5c) as well as the nasolacrimal duct (Fig. 5d) which connects the nasal and orbital cavities. When allergic rats were treated with  $\gamma$ T, allergen-induced eosinophilic inflammation into pulmonary and nasal airways was significantly attenuated by 30–60%.

### Gene expression

Lung lobe expression of IFN $\gamma$  eightfold, IL-4 (2.7-fold), IL-5 (fourfold), and IL-13 (12-fold) was induced by airway challenge to OVA compared with non-allergic controls (Table 4). Treatment with  $\gamma$ T augmented IFN $\gamma$  expression 2.5-fold in lungs from allergic rats, but had no effect on OVA-induced expression of ILs. In the nasal septum, by comparison, allergen-induced expressions of IFN- $\gamma$  3.4-fold, IL-4 (3.3-fold), IL-5 (2.7-fold), and IL-13 (1.5-fold) were all attenuated by  $\gamma$ T treatment.

### Discussion

Our results describe a spectrum of potentially therapeutic effects by  $\gamma$ T on allergen-induced inflammatory and epithelial cell responses in the airways of allergic rodents.

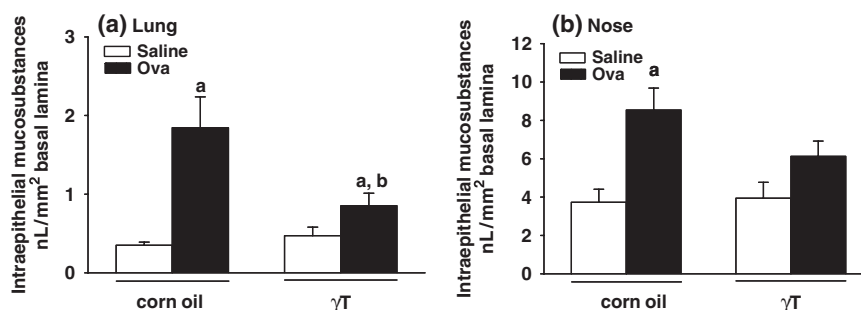


Fig. 4. Effect of  $\gamma$  tocopherol ( $\gamma$ T) on allergic mucous cell metaplasia in pulmonary and nasal airways. Rats were sensitized and challenged with saline or ovalbumin (OVA), and given corn oil (CO) or  $\gamma$ T. Intraepithelial mucosubstances were determined in (a) pulmonary conducting airways and (b) the nasal septum. a, significantly different from respective group challenged with saline. b, significantly different from respective group given CO.  $n = 7$ /group;  $P < 0.05$ .

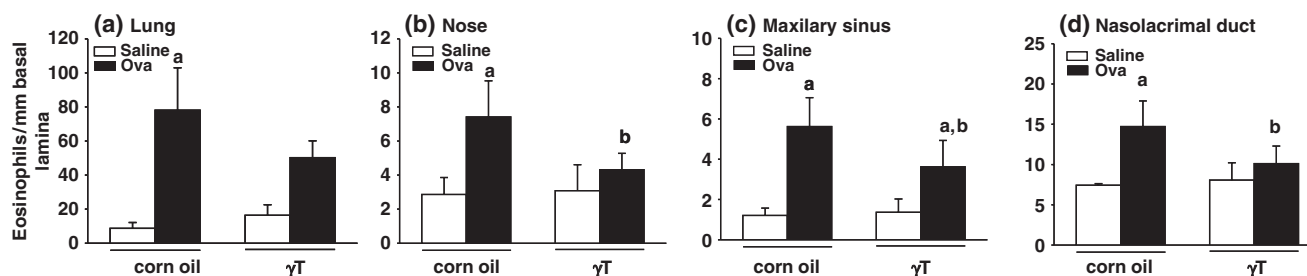


Fig. 5. Effect of  $\gamma$  tocopherol ( $\gamma$ T) on eosinophil infiltration in pulmonary and nasal tissues. Rats were challenged with saline or ovalbumin (OVA), and given corn oil (CO) or  $\gamma$ T. Eosinophilic inflammation was evaluated in (a) pulmonary conducting airways, (b) nasal septum, (c) maxillary sinus and (d) nasolacrimal duct. a, significantly different from respective group challenged with saline. b, significantly different from respective group given CO.  $n = 7$ /group;  $P < 0.05$ .

**Table 4.** Effect of  $\gamma$ -tocopherol on gene expression in lung lobe and nasal septum

| Experimental group  | Sal/vehicle     | Sal/ $\gamma$ T | OVA/vehicle        | OVA/ $\gamma$ T      |
|---------------------|-----------------|-----------------|--------------------|----------------------|
| Lung IFN- $\gamma$  | 1.00 $\pm$ 0.05 | 1.13 $\pm$ 0.17 | 8.47 $\pm$ 2.57 a  | 21.13 $\pm$ 8.43 a,b |
| IL-4                | 1.00 $\pm$ 0.18 | 1.00 $\pm$ 0.19 | 2.87 $\pm$ 0.34 a  | 3.5 $\pm$ 1.23 b     |
| IL-5                | 1.00 $\pm$ 0.21 | 0.87 $\pm$ 0.10 | 3.85 $\pm$ 0.61 a  | 3.22 $\pm$ 0.69 b    |
| IL-13               | 1.00 $\pm$ 0.15 | 1.16 $\pm$ 0.13 | 11.99 $\pm$ 2.90 a | 10.52 $\pm$ 2.88 b   |
| Nasal IFN- $\gamma$ | 1.00 $\pm$ 0.45 | 0.66 $\pm$ 0.25 | 3.39 $\pm$ 0.70 a  | 1.53 $\pm$ 0.37 b    |
| IL-4                | 1.00 $\pm$ 0.16 | 1.76 $\pm$ 0.26 | 3.34 $\pm$ 0.66 a  | 1.51 $\pm$ 0.23 b    |
| IL-5                | 1.00 $\pm$ 0.17 | 1.26 $\pm$ 0.15 | 2.73 $\pm$ 0.15 a  | 1.42 $\pm$ 0.30 b    |
| IL-13               | 1.00 $\pm$ 0.42 | 0.77 $\pm$ 0.17 | 1.50 $\pm$ 0.48 a  | 0.45 $\pm$ 0.12 b    |

a, significantly different from respective group challenged with saline,  $P < 0.05$ .

b, significantly different from respective group given corn oil,  $P < 0.05$ .

Sal, saline; OVA, ovalbumin.

Treatment with  $\gamma$ T attenuated inflammatory cell recruitment, cytokine gene expression, eicosanoid productions, epithelial cell metaplasia and the overproduction of mucus. Furthermore, therapeutic efficacy was seen with an acute dosing protocol, and protection in the entire respiratory tract from nasal and sinus airways to the deep lung. This report is the first to describe broad and comprehensive anti-inflammatory effects of  $\gamma$ T in allergic airways and suggests that it might be an effective adjunct or alternative medicine for use by patients with AR and asthma.

We have previously described the association of both eosinophils and neutrophils in the initiation and progression of allergic MCM [24]. In the present study, eosinophilic infiltrates that were dramatically increased in pulmonary and nasal tissues and in airspaces (via lavage) were blocked by treatment with  $\gamma$ T. Potential eosinophil-derived mediators known to induce MCM include cysLTs and IL-13 [25, 26]. In allergic rats, we found that  $\gamma$ T decreased IL-13 expression in the nose, but not the lung lobes of allergic rats, and that BALF cysLTs were mildly attenuated by treatment. Besides eosinophils, IgE-activated mast cells are a primary source of cysLTs in allergic airways. It is possible that in addition to blocking eosinophil recruitment,  $\gamma$ T may interfere with the activation of mast cells and may inhibit the production of inflammatory mediators (e.g. tryptase, histamine, cysLTs) as well as specific mucogenic stimuli.

Oxidative stress has been implicated in the severity of allergic airways symptoms, and oxidant pathways, specifically the production of reactive oxygen species (ROS), activate mucin gene transcription and MCM [27]. Oxidant pathways and damage to critical cellular lipids and proteins can be neutralized or repaired by  $\alpha$ T and  $\gamma$ T, both of which can directly scavenge lipid peroxyl radicals. But  $\gamma$ T is relatively less potent against many ROS compared with  $\alpha$ T [28], and even the clinical efficacy of  $\alpha$ T intervention against oxidative stress that may occur during asthma and AR is questionable [11, 29]. Therefore, it is unlikely that the inhibitory effects we document for  $\gamma$ T, a

relatively weaker ROS quencher than  $\alpha$ T, are due predominantly to its capacity to neutralize oxygen-derived free radicals.

Rather than targeting ROS, the protective effects of  $\gamma$ T could be mediated in part by the neutralization of reactive nitrogen species, such as nitric oxide and peroxynitrite, which can damage cellular lipids, proteins, and nucleotides. Eosinophils and neutrophils are primary sources of reactive nitrogen species during allergic inflammation, and their production has been implicated in the nitration and inactivation of key anti-inflammatory and protective enzymes, including catalase, superoxide dismutase and glutathione transferases [6]. Furthermore, nitric oxide can stimulate mucin gene expression, protein synthesis and secretion in airway epithelial cells [30, 31]. Unlike  $\alpha$ T,  $\gamma$ T is a highly efficient quencher of nitric oxide and peroxynitrite *in vitro* [32], and we have shown in laboratory rodents that  $\gamma$ T inhibits protein nitration during systemic inflammation [33]. It is reasonable therefore to speculate that  $\gamma$ T could provide protection from nitrosative stress and mucous cell responses that occur in allergic airways with eosinophilia such as we describe in our model. We observed a reduction in BALF  $\gamma$ T in allergic rats, which supports the possibility for the consumption of  $\gamma$ T by NO in allergic airways. This possibility is highly speculative, however, as we did not measure BALF NO or other reactive species, including nitrated  $\gamma$ T.

Clearly, the potential for eosinophil-derived NO is already diminished by the lower numbers of airway eosinophils after  $\gamma$ T treatment. However, activated neutrophils are also a potent source of NO during inflammation. We documented a significant accumulation of BALF neutrophils in our acute model, which were not attenuated by  $\gamma$ T treatment ( $P = 0.067$ ). Although not considered a classic T-helper type 2 (Th2) response, acute neutrophilic inflammation is often reported in allergic rodent models, and neutrophils are present in sputum and biopsies from asthmatics [34, 35].

Using  $\gamma$ T in models of non-allergic inflammation, we have previously described its ability to inhibit COX and

lipoygenase (LOX) activities, depressing the production of PGE<sub>2</sub> and LTB<sub>4</sub>, respectively [22, 23]. In many asthma studies, both endogenous and supplemented PGE<sub>2</sub> renders protection from adverse responses, due both to bronchodilation and by inhibition of alternative arachidonate-derived production of inflammatory leukotrienes. However, the potential actions of PGE<sub>2</sub> in asthma are very complex, due to variation in the location and functions of the four receptors (EP1–4) that bind this eicosanoid. For example, EP1 and EP3 receptors have pro-inflammatory actions, and have been shown to increase airway reactivity, decrease the bronchodilator function of the  $\beta$ 2-adrenergic receptor and enhance mast cell degranulation. In contrast, EP2 and EP4 exert anti-inflammatory actions on macrophages and mast cells. However, EP2 and EP4 have also been reported to have potent actions on dendritic cell maturation and chemotaxis, enhancing allergen-induced Th2 inflammation. Consistent with this idea is the observation that PGE<sub>2</sub> production by dendritic cells from asthmatics is increased relative to that in cells from non-asthmatic volunteers. Thus, PGE<sub>2</sub> could contribute to allergen-induced inflammation via actions on airway antigen-presenting cells [36, 37].

We also see decreased LTB<sub>4</sub> production by  $\gamma$ T, suggesting suppression of both COX and LOX activities during allergy. A critical role for LTB<sub>4</sub> has recently been described for the recruitment and activation of IL-13-producing T cells [38]. Both IL-13 and cysLTs are central mediators for inflammatory cell activation and epithelial responses during allergy, including mucin gene expression [25, 39]. Taken together, inhibition of both COX and LOX by  $\gamma$ T likely decreases a number of mediators derived from arachidonic acid that would promote allergen-induced inflammation.

Previous allergic airway intervention studies with vitamin E supplements have used  $\alpha$ T, which is the predominant form of vitamin E in mammalian tissues and for which the recommended dietary allowance (RDA) has been established. For example, in a 6-week study using daily supplements of 250 mg  $\alpha$ T (16 times RDA), asthmatics showed no improvement in airway function or frequency in bronchodilator use compared with placebo [11]. Doses of 800 mg  $\alpha$ T/day (50 times RDA) in patients with AR failed to decrease symptom frequency or medication use during the pollen season [13]. In a separate study of rhinitic patients, daily supplements with 267 mg  $\alpha$ T/day had no effect on symptom severity or serum IgE and lipid peroxides in response to allergen provocation [29].

Lack of efficacy by  $\alpha$ T was also recently reported in a rodent model where 400 mg/kg body weight/day of  $\alpha$ T for 10 days to sensitized rats failed to reduce allergen-induced pulmonary lesions or airway hyperreactivity [12]. It is notable in that study that dosing with tocopherol occurred after sensitization but before allergen challenge. Conversely, some animal studies demonstrate that  $\alpha$ T can be

effective against allergic responses if added to the diet before sensitization to allergen. When  $\alpha$ T feeding begins several weeks before immunization, subsequent airway allergen provocation responses of cytokine production, inflammatory cell recruitment, airway hyperreactivity, and oxidative stress can be inhibited [40, 41]. Therefore,  $\alpha$ T might specifically inhibit the sensitization pathways (e.g. antigen presentation, clonal expansion, IgE production), which would render animals insensitive to re-challenge to antigen. Similar relationships can be extrapolated from epidemiological studies, where non-asthmatics tend to ingest more tocopherols and antioxidants in their diets than asthmatics [19]. Taken together, results from animal models agree with human studies to suggest that, while potentially efficacious against sensitization to allergen,  $\alpha$ T provides little therapeutic value for acute airway exacerbations in subjects with pre-existing AR or asthma.

Our animal model is limited to acute allergic responses (inflammatory and mucous cell responses and gene expression) that occur within 24 h after two airway provocations with allergen. Several challenges to rodents over a week or more can produce chronic pathological alterations including smooth muscle hyperplasia, myofibroblasts recruitment, collagen and matrix depositions, and fibrosis, which are features not always seen in human asthma. We have also not addressed the potential effects of  $\gamma$ T on airway hyperreactivity. Future studies will examine the effects of  $\gamma$ T in pre-existing allergic airways and on functional responses to airway provocation to allergen.

We based our dosing regimen for  $\gamma$ T on our previous results that compared tocopherols in a non-allergic model of systemic inflammation [22, 23]. Specifically, we found that  $\gamma$ T was more effective than  $\alpha$ T against acute inflammatory responses. Pharmacological doses of 100 mg/kg  $\gamma$ T were used in our study, and our preliminary data in the same animal model suggest that anti-allergic effects of  $\gamma$ T may occur as low as 10 mg/kg (data not shown). Although the doses appropriate to human subject need to be established, it should be noted that the bioavailability of  $\gamma$ T in human tissues is much higher than that in rodents; thus, it has been documented that concentrations of  $\gamma$ T in human skin, adipose and muscle tissue were 6–60-fold greater than those in laboratory rodents [42, 43].

Lastly,  $\gamma$ T has been shown in cell culture systems to up-regulate peroxisome proliferator activated receptor gamma (PPAR $\gamma$ ), a nuclear transcription factor that regulates gene expression [44, 45]. PPAR $\gamma$ -agonists possess a broad array of anti-inflammatory effects in the asthmatic lung, including modulation of eosinophil survival and activation [46], and epithelial mucin production [47]. It is possible that the relatively rapid effects of  $\gamma$ T against allergen-induced inflammation could be mediated by an acute activation of PPAR $\gamma$  and by inhibition of COX and

LOX expression. By comparison, inhibition of nitration reactions by  $\gamma$ T may require repeated dosing in order to achieve optimal tissue levels. Together, these three mechanisms may provide a protective triad for the comprehensive therapeutic effects of  $\gamma$ T we have documented against inflammatory and epithelial cell responses in both pulmonary and nasal allergic airways.

In summary, we have used an animal model of allergic airways disease to describe novel anti-inflammatory effects of  $\gamma$ T after exposure to allergen. Therapeutic effects of  $\gamma$ T were seen with a relatively acute dosing regimen and protected the entire length of the respiratory tract. Based on these animal observations, phase I studies examining the safety and efficacy of  $\gamma$ T in persons with AR and asthma are currently underway.

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