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Isoforms of Vitamin E Have Opposing Immunoregulatory Functions during Inflammation by Regulating Leukocyte Recruitment

Sergejs Berdnikovs, Hiam Abdala-Valencia, Christine McCary, Michelle Somand, Rokeisha Cole, Alex Garcia, Paul Bryce, and Joan M. Cook-Mills

Reports indicate contradictory outcomes for anti-inflammatory functions of the α-tocopherol isoform of vitamin E in clinical studies of asthma and atherosclerosis. These seemingly disparate clinical results are consistent with novel unrecognized properties of isoforms of vitamin E reported in this study. We demonstrate that the isoform D-γ-tocopherol elevates inflammation in experimental asthma. Moreover, D-γ-tocopherol, at as little as 10% the concentration of D-α-tocopherol, ablates the anti-inflammatory benefit of the D-α-tocopherol isoform. A mechanism for these opposing immunoregulatory functions of purified tocochromanols at physiological concentrations is not through modulation of expression of several cytokines, chemokines, or adhesion molecules, but is, at least in part, by regulation of endothelial cell signals during leukocyte recruitment. These opposing regulatory functions of vitamin E isoforms have impact on interpretations of vitamin E studies. In summary, our studies with purified tocochromanol isoforms alter our understanding of vitamin E regulation of vascular function and asthma. The Journal of Immunology, 2009, 182: 4395–4405.

Leukocytes are recruited from the blood during inflammation. We have reported that during this recruitment, VCAM-1 activates signals in endothelial cells that are required for active participation of the endothelial cell during leukocyte transendothelial migration in vivo and in vitro (1–4). Thus, because VCAM-1 regulates leukocyte recruitment and vascular function during inflammation and because VCAM-1 signals in endothelial cells are mediated through reactive oxygen species, we hypothesized that isoforms of the antioxidant vitamin E regulate endothelial cell function during leukocyte recruitment, including VCAM-1-dependent recruitment of eosinophils in asthma.

Patients with asthma have low levels of the vitamin E isofor m α-tocopherol (5–8). Therefore, an increase in vitamin E may be necessary to promote optimal health in combination with other regimens to treat asthma. The α-tocopherol form of vitamin E has been suggested to exert anti-inflammatory actions and has been shown to be effective in reducing asthma in studies in some European countries (Finland and Italy) (9, 10). Disappointingly, vitamin E trials in the U.S. and The Netherlands using α-tocopherol supplements have failed to show benefit in asthma (10–13). Similar to studies of asthma, there are contradictory outcomes for tocopherol benefit in clinical trials of atherosclerosis (reviewed in Refs. 14 and 15). These asthma and atherosclerosis clinical studies have focused on one form of vitamin E, α-tocopherol, without analysis of other isoforms of vitamin E that are consumed in diets. The most abundant forms of vitamin E are α-tocopherol and γ-tocopherol. γ-Tocopherol is the major form of vitamin E in the diet of Americans, but is not in abundance in most European diets (16) because D-γ-tocopherol is abundant in soy and vegetable (often soy) oil in the American diet (17). Consistent with this, several reports indicate that plasma levels of γ-tocopherol in Americans and in Dutch are two to six times higher than most Europeans (reviewed in Ref. 16). We suggest that the seemingly contradictory outcomes for α-tocopherol supplements on regulation of inflammation are the result of the sum of effects of different mixtures of tocochromanol isoforms acquired from the diet and present in tissues.

Dietary tocopherols are taken up from the intestine and transported via the lymph to the blood and then to the liver. In the liver, α-tocopherol transfer protein (αTTP)4 preferentially transfers α-tocopherol to plasma lipoproteins, resulting in retention of γ-tocopherol in tissues at 10% that of α-tocopherol (17). Nevertheless, αTTP carries significant quantities of γ-tocopherol into circulation (17). Tocopherols in plasma lipoproteins are transferred to cells by plasma phospholipid transfer protein, scavenger receptors, or the lipoprotein lipase pathway (18). After uptake, the tocopherols are found in cell membranes because they are lipid vitamins. We have studied the modulation of leukocyte recruitment in vivo and in vitro by the two major vitamin E isoforms.

Using purified forms of vitamin E at physiological concentrations, we report in this study the novel finding that γ-tocopherol elevates inflammation in experimental asthma. Importantly, D-γ-tocopherol, at 10% the tissue concentration of D-α-tocopherol, opposes D-α-tocopherol inhibition of inflammation in vivo and in vitro. References are included for further details.

4 Abbreviations used in this paper: αTTP, α-tocopherol transfer protein; BAL, bronchoalveolar lavage; CBA, cytokine bead assay; Penh, enhanced pause; PKC, protein kinase C.
vitro. The mechanism of action of these tocopherols is through a direct regulation of endothelial cell signals during leukocyte recruitment. These data have important implications on interpretations of reports on vitamin E, on the study of the increased rate of asthma in the last 40 years in Americans, and on design of future studies with vitamin E.

Materials and Methods

Animals

BALB/c mice were from The Jackson Laboratory. The studies are approved by the Northwestern University institutional review committee for animals.

Cells

The endothelial cell line mHEVa was cultured, as previously described (2, 19). Spleen cells were prepared from freshly isolated male BALB/c mouse spleens (19). Where indicated, spleen RBC were lysed by hypotonic shock (19).

**Tocopherol reagents**

Ethoxylated castor oil was composed of polyethoxylated castor oil (PEG35; BASF Chemical), 20% ethanol, and 1% benzyl alcohol. Vital E-300 IU (Schering-Plough) was composed of α-tocopherol (99% pure) in ethoxylated castor oil. γ- and δ-Tocopherol (Sigma-Aldrich) was used in all in vitro studies. We confirmed the purity of these tocopherols by HPLC with electrochemical detection, as described below. The purified tocopherols were diluted in ethoxylated castor oil for the in vitro studies. The purified tocopherols were diluted in DMSO (0.2%) for the in vitro studies. There was no effect of DMSO or the tocopherols on viability of the endothelial cells or leukocytes as determined by trypan blue exclusion (data not shown). The following oils were purchased from local markets: sunflower oil (President’s Choice), soy oil (Crisco), canola oil (Crisco), corn oil (Mazola), olive oil (Colavita or Philip Barrio), and Turkish olive oil (Eskigiski and Incecek; Tariszetin).

**OVA/Tocopherol administration and inflammation**

BALB/c female mice were maintained on chow diet (Fig. 1B). The mice were sensitized by i.p. injection (200 μl) of OVA grade V (10 μg/μl) or saline/albumin on days 0 and 7 (1). On days 13–20, these mice received daily s.c. injections (50 μl) of 2 mg of natural tocopherol/ethoxylated castor oil (20) or ethoxylated castor oil alone. This daily 2 mg of natural tocopherol is equivalent to the amount of tocopherol that mice consume from diets (4-g diet consumed/day per 500 mg of α-tocopherol/kg diet) that are commonly used to elevate tissue tocopherols in mice (21, 22). The mice received 2 mg of α-tocopherol (Vital E300; Schering-Plough), 2 mg of δ- and γ-tocopherol (Sigma-Aldrich), 2 mg of α-tocopherol plus 2 mg of δ- and γ-tocopherol or the vehicle ethoxylated castor oil. On days 16, 18, and 20, the mice were challenged with intranasal OVA fraction VI (150 μg) in saline or saline alone (1). On day 21, the mice were analyzed for body weight, leukocyte levels, and inflammation. Bronchoalveolar lavage (BAL) cells and blood eosinophils were stained and counted, as previously described (1). VCAM-1 expression and eosinophils in frozen lung tissues were examined by labeling with anti-VCAM-1 or labeling eosinophil granules with anti-major basic protein Abs, respectively, as previously described (1). OVA-specific IgE was determined by ELISA, as previously described (23).

**Tocopherol measurement**

Lungs that are perfused blood free were weighed and homogenized in absolute ethanol with 5% ascorbic acid on ice. The internal standard tocol was extracted with an equal volume of hexane with 0.37 weight percent butylated hydroxytoluene to prevent oxidation and increase recovery of tocopherol. The samples were vortexed and then centrifuged for 10 min at 9000 × g at 4°C. The hexane layer was dried under nitrogen and stored at −20°C. The samples were reconstituted in methanol, and then tocopherols were separated using a reverse-phase C18 HPLC column (Hewlett-Packard) and HPLC (Waters) with 99% methanol-1% water as a mobile phase with detection with an electrochemical detector (potential 0.7 V; Waters).

**Cytokines, chemokines, and PGE2**

The BAL supernatants were tested for levels of cytokines using the Th1/Th2 cytokine bead assay (CBA) kit (BD Biosciences). Eotaxins were determined by quantitative PCR from lung tissue. Total RNA was isolated from 50–100 mg of lung tissue using the Qiagen RNeasy Fibrous Tissue Mini Kit. cDNA was prepared using a SuperScript II RNase H-Reverse Transcriptase Kit (Invitrogen) and analyzed by PCR on an ABI 7300 Thermal Cycler (Applied Biosystems). Taqman probes and Taqman Universal Master Mix were used as directed (Applied Biosystems) for eotaxin 1 and 2. MCP-1 from endothelial cell culture supernatants was measured by CBA inflammation kit (BD Biosciences). The ethanol fraction from the lung extracts for tocopherol measurement described above was collected, dried under nitrogen, and suspended in the buffer of the High Sensitivity PGE2 ELISA Kit (LCG Bioscience).

**Airway responsiveness**

Lung responsiveness on day 21 of the OVA inflammation protocol (Fig. 1B) was determined using methacholine and whole body plethysmography, as previously described (24). The whole-body plethysmography on conscious, unrestrained mice was performed under continuous airflow conditions, and enhanced pause (Penh) was calculated (Buxco). Penh, obtained under continuous flow conditions, correlates well with specific airway resistance (25, 26).

**In vitro Transwell migration assay**

mHEVa cells were grown to confluence on Transwells with 12-μm pores (Costar). Spleen cell migration was performed, as previously described (19). The endothelial cell monolayers block nonspecific accumulation of spleen RBC in the lower Transwell chamber and block FITC-albumin diffusion (27). Spleen cell migration is stimulated by mHEVa cell secretion of the chemokine MCP-1 (28). The number of lymphocytes that migrate is linear from 0 to 24 h, followed by a plateau (27). Spleen cells that migrate are greater than 95% lymphocytes (27), and the percentage of cells that migrate is consistent with other in vitro models with endothelial cell lines or cytokine-activated microvascular endothelial cells (29, 30).

**In vitro cell association and migration assays with laminar flow**

A parallel plate flow chamber was used to examine leukocyte migration under conditions of laminar flow of 2 dynes/cm2 as previously described (2, 3). Leukocyte migration was examined at 15 min. The number of cells that were associated, but not migrated (phase light cells), at 15 min is low because in 15 min, the majority of nonmigrating cells roll off the monolayer of endothelial cells, as determined by microscopy (data not shown). Therefore, the number of spleen cells associated with the endothelial cells at 2 min of laminar flow are those cells that mediated cell-cell contact.

**Western blot for protein kinase C (PKC)α phosphorylation**

Cells were stimulated by cross-linking VCAM-1 with 24 mg/ml rat anti-mouse VCAM-1 (clone MVCAM-A; BD Pharmingen) plus 12 mg/ml goat anti-rat IgG Ab (cat. no. 3050-01; Southern Biotechnology Associates), as we previously described (2). The Abs were incubated together for 5 min and added to the endothelial cells. The tocopherols were added to medium from the endothelial cell monolayers so that there was no addition of fresh serum-containing medium that stimulates growth factor receptors. After 10 min, the optimal for VCAM-1 activation of PKCα (2), cells were lysed in SDS sample buffer (10% glycerol, 5% 2-ME, 3% SDS, 0.5 M Tris-HCl (pH 6.8), and 0.1% bromphenol blue), incubated on ice for 20 min, sonicated, boiled for 5 min, and centrifuged at 14,000 rpm for 5 min. These cell lysates were analyzed by 7.5% SDS-PAGE and semidry transferred to nitrocellulose membranes, according to manufacturer’s instructions (Bio-Rad) (12 mA for 40 min). The membranes were blocked in 5% nonfat dried milk in TBST (0.5% Tween 20 (TBST), 1× PBS, 0.1% 2-ME, and 0.5 M Tris-HCl (pH 6.8)), washed with TBST, and probed for β-actin (catalog no. ab8226; Abcam), followed by labeling with HRP-conjugated donkey anti-rabbit Ab (Amersham Biosciences) or HRP-conjugated goat anti-mouse IgG (Bio-Rad). Detection was by ECL (Amersham) and autoradiography. Densitometry of the bands was performed using Image J software (National Institutes of Health). The data are presented as the fold increase in the ratio of relative intensity of the band/the relative intensity of band for the loading control (total PKCα, or β-actin).
natural D-tocopherol was endogenously synthesized by mice (31). We used purified natural ascorbic acid, which recycles tocopherols because ascorbic acid rather than a mixture of the multiple racemic forms of tocopherols in vivo. For these studies, we used purified natural forms of the experimental asthma.

**Results**

Two forms of vitamin E have opposite effects on inflammation in experimental asthma

We examined whether tocopherols affected leukocyte recruitment in vivo. For these studies, we used purified natural forms of the tocopherols rather than mixtures of the multiple racemic forms of natural or synthetic tocopherols. We did not administer supplemental ascorbic acid, which recycles tocopherols because ascorbic acid is endogenously synthesized by mice (31). We used purified natural d-α-tocopherol or purified natural d-γ-tocopherol, which differ by one methyl group (Fig. 1A). Before treatment, we confirmed purity (>98%) and concentration of these tocopherols by HPLC with electrochemical detection (data not shown).

Briefly, female BALB/c mice were maintained on standard commercial rodent chow containing basal levels of tocopherols (32). We sensitized mice by i.p. injection of OVA/alum, administered tocopherol, and challenged with OVA, as in the timeline in Fig. 1B (1). These mice were examined for inflammation on day 21. To modulate the inflammation, daily s.c. injections of tocopherols (20) were administered after the Ag sensitization phase, but before the Ag challenge phase (Fig. 1B). This is especially important because asthmatics responding to Ag are already sensitized. Furthermore, a few days of s.c. administration of tocopherols were used in these studies rather than feeding for weeks with tocopherol-containing diets to change tissue tocopherol levels (33), so that tissue tocopherol levels were altered in the few days before Ag challenge. With dietary or with s.c. routes of tocopherol administration, the tocopherols are transported via the lymph to the blood and then to the liver, where they are transferred to lipoproteins that are then taken up by cells. Ethoxylated castor oil was used as vehicle (20) because it does not contain tocopherols and because it is used in drug formulations for viscous lipids because it has little side effects. The daily 2 mg of natural tocopherol is equivalent to the amount of tocopherol that mice consume from diets (4-g diet consumed/day × 500 mg of α-tocopherol/kg diet) that are commonly used to elevate tissue tocopherols in mice (21, 22). The body weights and lung weights of the mice in our studies were unaltered by the tocopherol treatment blocked the benefit of d-α-tocopherol so that, in the d-α- plus d-γ-tocopherol-treated mice, there was an intermediate phenotype that was not significantly different from leukocyte numbers in OVA-stimulated vehicle-treated mice (Fig. 3, A and B). Similarly, in lung tissue, d-γ-tocopherol treatment elevated

**Statistics**

Data were analyzed by a one-way ANOVA, followed by Tukey’s or Dunnett’s multiple comparisons test (SigmaStat; Jandel). Presented are the means ± SEs.

**FIGURE 1.** Schematic for tocopherol treatments during Ag activation of experimental asthma. A, Structure of natural d-α-tocopherol (d-α-T) and natural d-γ-tocopherol (d-γ-T) that differ by one methyl group (arrows). B, Schematic of time line for tocopherol and OVA treatments. i.n., Intranasal. n = 8–10 animals per group.

**FIGURE 2.** Tocopherol levels and mouse weight on day 21. A, Mouse body weight. B, Wet weight of right lung lobes. C and D, Mice were treated with tocopherols, as in Fig. 1B. Plasma was collected and lungs were perfused free of blood. Plasma or lung tocopherol levels were measured by HPLC. Column A, Mice on Chow diet were administered vehicle. Column B, Mice on Chow diet were administered d-α-tocopherol (αT). Column C, Mice on Chow diet were administered d-γ-tocopherol (γT). Column D, Mice on Chow diet were administered both αT and γT. n = 8–10 animals per group. *p < 0.05 compared with vehicle control.
lungs, there were sufficient numbers of eosinophils in the blood available for migration (Fig. 3E). Furthermore, OVA-specific IgE Abs, which are induced during the OVA sensitization phase of experimental asthma, were not affected by these tocopherols (Fig. 3F). This lack of an effect on IgE is consistent with the tocopherol administration after the sensitization phase. In summary, α-tocopherol and γ-tocopherol had opposing regulatory functions during leukocyte accumulation in the lung in response to OVA.

In asthma, there is increased lung responsiveness to methacholine. Therefore, we examined responsiveness to methacholine inhalation by whole-body plethysmography (24) because this method of measuring lung function has the advantage of analysis of large numbers of animals per day and because whole-body plethysmography correlates well with airway function when performed under continuous flow conditions (25, 26). As expected, vehicle/OVA-treated mice had an increase in responsiveness as calculated as Penh in the BALB/c mice compared with saline-treated control groups (Fig. 3G). This increase was completely ablated by administration of α-tocopherol (Fig. 3G, filled arrow). Moreover, α-γ-tocopherol-elevated Penh (Fig. 3G, arrowhead) and δ-γ-tocopherol antagonized the inhibition by α-tocopherol (Fig. 3G, unfilled arrow). Thus, the tocopherol modulation of leukocyte accumulation in Fig. 3, A–D, is consistent with the α-tocopherol ablation of Penh and the α-γ-tocopherol elevation of Penh in Fig. 3G. In summary, isoforms of tocopherols had opposing regulatory functions on inflammation in experimental asthma.

**Tocopherol does not alter expression of several immune modulators of inflammation**

We determined whether a selected set of cytokines, chemokines, and adhesion molecules that regulate leukocyte infiltration in experimental asthma is altered with the tocopherol treatments of the mice. The cytokines IL-4 and IL-5, the chemokines eotaxin 1 and eotaxin 2, and the adhesion molecule VCAM-1 were examined because they stimulate eosinophil and lymphocyte infiltration. The cytokines IL-2 and IFN-γ were examined because they down-regulate signals for OVA-induced leukocyte infiltration. As expected, OVA-treated mice had increased expression of the Th2 cytokines IL-4 and IL-5, the chemokines eotaxin 1 and eotaxin 2, and the adhesion molecule VCAM-1 (Fig. 4, A–E). However, the tocopherols did not cause significant changes in the expression of these mediators (Fig. 4, A–E). Moreover, the tocopherols did not switch the Th2 cell response to a Th1 response because, in the tocopherol-treated mice, there was no induction of expression of the Th1 cytokines IFN-γ and IL-2 (Fig. 4, F and G). We also examined PGE2 levels because a reduction of PGs from μM to nM concentrations is proinflammatory and because tocopherol has been reported to reduce PGE2 synthesis (34). However, there was no reduction in PGE2 in the α-γ-tocopherol-treated groups (Fig. 4H).
been reported that 40–100 μM concentrations of purified D-tocopherol or cytokines, we determined whether physiological concentrations of tocopherols without affecting expression of adhesion molecules, chemokine-dependent and chemokine-dependent leukocyte migration to examine leukocyte transendothelial migration in vitro.

Two forms of vitamin E have opposing regulatory functions during leukocyte transendothelial migration in vitro

Because in vivo, leukocyte trafficking was regulated by tocopherols without affecting expression of adhesion molecules, chemokines, or cytokines, we determined whether physiological concentrations of purified D-tocopherol or D-γ-tocopherol regulate the transendothelial migration event. For these studies, we used a well-developed in vitro endothelial cell model of adhesion molecule-dependent and chemokine-dependent leukocyte migration to examine leukocyte transendothelial migration. In this model, the migration of lymphocytes across the endothelial cell line mHEVs is dependent on the endothelial cell constitutive expression of VCAM-1 and the chemokine MCP-1 (19, 28).

To examine tocopherol function in vitro, we used physiological concentrations of purified tocopherols (2–80 μM) because it has been reported that 40–100 μM α-tocopherol modulates cell functions in vitro (35–40) and plasma α-tocopherol is from 35 (41) to 60 μM with supplemental intake of 800 IU α-tocopherol/day (42). We incubated endothelial cells overnight with tocopherols because it has been reported that endothelial cells take up tocopherols from culture medium after overnight incubation (43). This 18-h incubation of the endothelial cells with 80 μM D-α-tocopherol raised the endothelial cell concentration of α-tocopherol 5-fold compared with the DMSO control group (Fig. 5, A and C), which is similar to the 5-fold increase in α-tocopherol in lungs of D-α-tocopherol-treated mice (Fig. 2C, column B). In addition, incubation of endothelial cells for 18 h with 2–5 μM D-γ-tocopherol raised the endothelial cell concentration of γ-tocopherol (Fig. 5, B and C) to that in the lung tissue of D-γ-tocopherol-treated mice (Fig. 2C, column C). It is noted that the perfused lung tissue used to measure in vivo concentrations includes all cells of the lung tissue. Nevertheless, D-α-tocopherol inhibited leukocyte migration in a Transwell migration assay (Fig. 6A), whereas D-γ-tocopherol significantly increased leukocyte migration (Fig. 6B). In these overnight transendothelial migration assays, the tocopherols were left in the culture medium. The vehicle DMSO (0.02%) did not affect leukocyte migration (data not shown). The tocopherols and DMSO also did not affect cell viability as determined by trypan blue exclusion (data not shown), and did not affect endothelial cell morphology as determined by microscopy (data not shown). The tocopherols did not overtly affect endothelial cell monolayer integrity because there was no nonspecific migration of the internal control spleen RBC (data not shown). Interestingly, in Fig. 6C, treatment with D-γ-tocopherol ablated the D-α-tocopherol inhibition of spleen leukocyte migration in the Transwell assay, resulting in an intermediate phenotype not different from vehicle-treated cells.

These tocopherols did not alter the VCAM-1 dependence of the leukocyte migration, as demonstrated by treatment with blocking anti-VCAM-1 Abs (Fig. 6D). Furthermore, there was no effect of these tocopherols on endothelial cell expression of VCAM-1, as determined by immunolabeling VCAM-1 on the endothelial cells and flow cytometry (Fig. 6E). Thus, in vitro (Fig. 6E) and in vivo (Fig. 4E), these tocopherols did not alter VCAM-1 expression. There was also no effect of tocopherols on MCP-1 expression (Fig. 6F). In summary, purified D-α-tocopherol and D-γ-tocopherol at
physiological concentrations in vitro had opposing functions during leukocyte transendothelial migration that paralleled the in vivo regulatory functions of these tocopherols.

A mechanism for the tocopherol modulation of inflammation is through direct regulation of endothelial signals during leukocyte transendothelial migration

To first examine the effects of tocopherols on the leukocyte during transendothelial migration, mice were treated with 2 mg of these tocopherols in vivo daily for 4 days, and then the spleen cells were added to the upper chamber of the Transwells to examine cell migration. A, Dose curve of αT. B, Dose curve of γT treatment. C, Dose curve of γT effects on αT (80 μM)-treated cells. D, Endothelial cells were treated overnight with tocopherol, and then with the presence or absence of blocking anti-VCAM-1 Abs, spleen cell migration was determined. Anti-VCAM-1 Abs were added every 4 h, as previously described (19). E and F, Expression of VCAM-1 and MCP-1 by endothelial cells. E, Mean fluorescent intensity of anti-VCAM-1 or isotype Ab control-labeled cells. F, MCP-1 in mHEVa culture supernatant. G–K, Tocopherol pretreatment of cells, followed by washing before the start of the leukocyte migration assay with physiological laminar flow. G, Mice were treated with 2 mg of tocopherol/day or vehicle (ethoxylated castor oil) for 4 days. Spleens were collected. RBC were lysed, and leukocyte tocopherol was determined. H, Spleen leukocytes from untreated mice or spleen leukocytes from mice treated with tocopherols as in G were added to the endothelial cells and examined for transendothelial migration at 15 min under physiological laminar flow. I, Spleen leukocytes from untreated mice or spleen leukocytes from mice treated with tocopherols as in G were added to the endothelial cells and examined for association with the endothelial cells at 2 min under physiological laminar flow. J and K, 85% confluent monolayers of mHEVa cells on slides were treated overnight with the tocopherols at the concentrations indicated to achieve physiological concentrations of tocopherols (Fig. 5), and then the endothelial cells were washed before addition of spleen cells. Spleen cells were isolated from untreated mice, the spleen RBC were lysed, and these spleen leukocytes were added to the endothelial cells to examine leukocyte transendothelial migration under physiological laminar flow. J, Leukocyte transendothelial migration at 15 min under laminar flow. K, Leukocyte association with endothelial cells at 2 min under laminar flow. n = 3–5. *, p < 0.05 compared with DMSO vehicle-treated controls. DMSO (0.02%) did not affect migration (data not shown).
DMSO-treated/anti-VCAM-stimulated group. PKCα, which we previously reported (2). The cells were lysed and examined for PKC activity (45). To examine modulation of VCAM-1 signals, endothelial cells were pretreated overnight with the tocopherols, as in Materials and Methods. These concentrations of α-tocopherol (80 µM) and γ-tocopherol (2 µM) were optimal for their inhibition or enhancement, respectively, of VCAM-1 activation of PKCα (data not shown). Shown are representative blots and data from five experiments. *, p < 0.05 compared with nonstimulated groups. **, p < 0.05 compared with nonstimulated groups, and p < 0.07 compared with DMSO-treated/anti-VCAM-stimulated group.

FIGURE 7. Tocopherols regulate VCAM-1 activation of endothelial cell PKCα. Endothelial cells were pretreated overnight with physiological concentrations of tocopherols (80 µM α-tocopherol and/or 2 µM γ-tocopherol), as in Fig. 5. Endothelial cell VCAM-1 was stimulated with anti-VCAM-1 and a secondary Ab for 10 min. Phosphorylation of PKCα-Thr638 was examined by Western blot, developed using an ECL kit, and analyzed by densitometry, as in Materials and Methods. These concentrations of α-tocopherol (80 µM) and γ-tocopherol (2 µM) were optimal for their inhibition or enhancement, respectively, of VCAM-1 activation of PKCα (data not shown). Shown are representative blots and data from five experiments. *, p < 0.05 compared with nonstimulated groups. **, p < 0.05 compared with nonstimulated groups, and p < 0.07 compared with DMSO-treated/anti-VCAM-stimulated group.

(d Data not shown). Furthermore, tocopherol pretreatment of the endothelial cells did not alter spleen leukocyte association with the endothelial cells (Fig. 6K), indicating that leukocytes were bound, but did not migrate. In summary, D-α-tocopherol and D-γ-tocopherol had regulatory functions in endothelial cells during leukocyte transendothelial migration.

Next, we determined whether the tocopherols regulate signals in endothelial cells that are required for leukocyte transendothelial migration. The adhesion molecule VCAM-1 on endothelial cells activates NADPH oxidase generation of reactive oxygen species that then directly oxidize and activate PKCα (2). This PKCα activity is required for this leukocyte transendothelial migration (2).

It has been reported that tocopherols scavenge reactive oxygen species and can inhibit reactive oxygen species-independent PKCα activity (45). To examine modulation of VCAM-1 signals, endothelial cells were pretreated overnight with the tocopherols, as in Fig. 6J. The culture medium was removed and replaced with culture medium from nontreated endothelial cells to avoid stimulation with growth factors from fresh serum. VCAM-1 on the endothelial cell monolayers was stimulated with anti-VCAM-1 plus a secondary Ab for 10 min, as we previously described (2). Control Ab binding (anti-CD98) does not activate PKCα (data not shown), as we previously reported (2). The cells were lysed and examined for the active form of PKCα, phospho-Thr638 PKCα by Western blot. The overnight treatment with tocopherols did not alter total PKCα expression (Fig. 7), D-α-Tocopherol significantly inhibited VCAM-1 activation of PKCα, and D-γ-tocopherol ablated this inhibition (Fig. 7). Thus, these tocopherols have opposing regulatory functions on VCAM-1 activation of PKCα.

In summary, D-α-tocopherol and D-γ-tocopherol directly regulated endothelial cell signals during leukocyte transendothelial migration. This occurred without altering endothelial cell adhesion molecule expression or chemokine expression. In contrast, pretreating leukocytes with these tocopherols did not affect leukocyte transendothelial migration, indicating that tocopherols did not directly regulate leukocyte function during migration.

Tocopherol concentrations in dietary oils

Because oils are significant sources of dietary tocopherols, we determined tocopherol concentrations in common cooking oils (Fig. 8A). Tocopherols were extracted from commercial oils and measured by HPLC with electrochemical detection, as described in Materials and Methods. Soy oil, the common vegetable oil consumed in the U.S., was rich in γ-tocopherol and had low levels of α-tocopherol compared with the other oils (Fig. 8A). Olive oils and corn oil had essentially undetectable γ-tocopherol and little α-tocopherol (Fig. 8A). In contrast, sunflower oil was rich in α-tocopherol and had little γ-tocopherol (Fig. 8A). Canola oil had intermediate levels of α-tocopherol and γ-tocopherol compared with the other oils (Fig. 8A). In summary, the concentrations of α-tocopherol and γ-tocopherol were considerably different among the oils tested.

Discussion

In this study, we demonstrate that the D-γ-tocopherol form of vitamin E elevates leukocyte recruitment to the lung in experimental asthma. In contrast, leukocyte recruitment is inhibited by elevation of tissue levels of the D-α-tocopherol form of vitamin E. The body weight and lung weights of the mice in our studies were unaltered by the D-α-tocopherol or D-γ-tocopherol treatments, which is consistent with previous reports for D-α-tocopherol consumption (33). Importantly, D-γ-tocopherol, at 10% the tissue concentration of D-α-tocopherol, blocks the D-α-tocopherol inhibition of leukocyte infiltration. This 10-fold difference in tissue levels of α-tocopherol vs γ-tocopherol of the tocopherol-treated mice is expected because there is preferential transfer of α-tocopherol in the liver by αTTP (17). The D-α-tocopherol inhibits and D-γ-tocopherol elevates leukocyte transendothelial migration by a direct regulatory function in endothelial cells. Moreover, D-γ-tocopherol blocks the D-α-tocopherol inhibition of leukocyte transendothelial migration in vitro. These opposing tocopherol regulatory functions in endothelial cells occur, at least in part, by tocopherol regulation of VCAM-1 activation of endothelial cell PKCα. We have previously

FIGURE 8. Tocopherol content in cooking oils and human plasma γ-tocopherol levels in several countries. A, Tocopherol in sunflower oil (President's Choice), soy oil (Crisco), canola oil (Crisco), corn oil (Mazola), olive oil 1 (Colavita), olive oil 2 (Incecik), olive oil 3 (Phillipo Bario), and olive oil 4 (Eskisigiki) as determined by HPLC/ECD. Shown are mean ± SEM. Bars without error bars indicate errors that are too small to be seen on the graph. B, Human plasma tocopherol levels in several countries (this panel was adapted from review (16)).
reported that the activation of PKCα is required for VCAM-1-dependent leukocyte migration (2). The tocopherols do not regulate the experimental asthma by modulation of cytokines, chemokines, or adhesion molecules. This tocopherol modulation of leukocyte infiltration without alteration of adhesion molecules, cytokines, or chemokines is similar to previous reports of in vivo inhibition of intracellular signals in endothelial cells without alteration of expression of these extracellular modulators of leukocyte trafficking (1, 46, 47). In summary, natural α-tocopherol and natural δ-γ-tocopherol differ in structure by only one methyl group, but at pathological concentrations, have opposing regulatory functions in endothelial cells that modulate inflammation. This is the first report on δ-γ-tocopherol elevation of inflammation and δ-γ-tocopherol modulation of δ-α-tocopherol function during inflammation. These results have important implications for the interpretation of clinical reports on vitamin E regulation of inflammation.

Published clinical studies on vitamin E and asthma focus on α-tocopherol. In contrast, the dietary contribution of γ-tocopherol on these clinical outcomes has not been reported. We and others have determined the levels of α-tocopherol and γ-tocopherol in dietary oils (Fig. 8A) (16, 48). The American diet is rich in γ-tocopherol found in soy oil, the major form of vegetable oil in the U.S. In contrast, γ-tocopherol is low in other oils (sunflower and olive oil) commonly used in European countries (Fig. 8A) (16, 48). Consistent with this, in the U.S. and The Netherlands, the average plasma γ-tocopherol level is 2–6 times higher than that of six European countries, including Italy (Fig. 8B) (16). The average plasma concentration of α-tocopherol is the same among the countries (16). Furthermore, clinical studies indicate that α-tocopherol supplementation of asthmatic patients is beneficial in Italy and Finland, but disappointingly, α-tocopherol is not beneficial for asthmatic patients in studies in the U.S. or The Netherlands (9–13). Our data on α-tocopherol plus γ-tocopherol treatment of mice had a similar outcome as that reported in the clinical studies in the U.S., in that there was little benefit of α-tocopherol for inflammation in the presence of elevated plasma γ-tocopherol. In our studies, the level of α-tocopherol is at the level of supplementation of α-tocopherol in mice, and the level of γ-tocopherol is at the level of γ-tocopherol elevation from dietary intake of γ-tocopherol. Therefore, differences in outcome of the clinical reports on vitamin E modulation of asthma in European countries and the U.S. may, in part, reflect the opposing regulatory functions of α- and γ-tocopherol forms of vitamin E consumed in diets and supplements. Although there are many other differences regarding the environment and genetics of the people in these countries, and it is acknowledged that other dietary factors, including unsaturated fatty acids, may modulate asthma (10, 49–53), the clinical data are consistent with our animal studies. Furthermore, the rate of asthma in several countries, including the U.S. and The Netherlands, has dramatically increased in the last 40 years (54–56). It is thought that there must be environmental factors contributing to this increase because it is too rapid for genetic changes. The prevalence of asthma is higher in the U.S. than Western Europe or Mediterranean countries (57). The World Health Organization has reported that the prevalence of asthma from 1950 to the present has increased in many countries, including countries with high rates of asthma, intermediate rates of asthma, or low rates of asthma (58). The increases in prevalence occur as countries assume western lifestyles (58). The dietary changes in the U.S. with increased consumption of γ-tocopherol in vegetable oil may, in part, be a contributing factor to changes in asthma prevalence. Therefore, because α-tocopherol levels are low in asthmatics (5–8) and because α-tocopherol can reduce inflammation, an increase in α-tocopherol in the presence of low γ-tocopherol may be necessary to promote optimal health in asthmatics in combination with other regimens to treat inflammation.

Tocopherols have been highly studied in other inflammatory diseases. We suggest that tocopherol isoform levels may affect the severity of other diseases with inflammation, including osteoarthritis and atherosclerosis. It has been reported that plasma γ-tocopherol is positively associated with osteoarthritis, whereas plasma α-tocopherol is negatively associated with osteoarthritis (59). In contrast, in another report on knee osteoarthritis, vitamin E supplementation (α-tocopherol) did not relieve symptoms, but they did not measure α-tocopherol or γ-tocopherol levels (60). With regards to coronary heart disease and stroke, the benefit of tocopherols is inconsistent among the studies (14, 15); furthermore, measurement of levels of both α-tocopherol and γ-tocopherol is commonly not reported (14, 15, 61–64). Studies of tocopherols and heart disease are also complex because different dietary oils not only contain different forms of tocopherols, but also contain different lipids that affect vascular function and heart disease. It has been reported that plasma γ-tocopherol levels are not associated with heart disease or in other reports are associated with an increase in relative risk for myocardial infarction (reviewed in Ref. 15). In contrast, α-tocopherol intake is either not associated with heart disease or, in other reports, is associated with reduced death from heart disease (14, 62–64). Therefore, although the clinical reports on heart disease are inconsistent, for those reports with an effect on heart disease, γ-tocopherol is associated with an increase, whereas α-tocopherol is associated with a decrease in parameters of heart disease. The opposing functions of tocopherols that we have observed alter the interpretation of clinical studies with mixed tocopherols in supplements and diets. Thus, future clinical studies of vitamin E regulation of inflammatory diseases should be systematically designed to examine opposing functions of the isoforms of vitamin E on inflammation.

Our data on tocopherol regulation of inflammation also alter interpretations of animal studies with tocopherols. First, in contrast to human diets, mouse chow contains low to no γ-tocopherol. In addition, many reports with animal studies indicate that vitamin E was administered to animals, but the form, source, and purity of tocopherols are not reported and the tissue levels of tocopherols after administration are sometimes not determined. In a report by Suchankova et al. (65), purified α-tocopherol was administered in soy oil by gavage, and they found no major effect of α-tocopherol on immune parameters and airway responsiveness in mice with experimental asthma. The soy oil vehicle used in their study contains an abundance of γ-tocopherol (Fig. 8A) and they did not measure tissue tocopherol levels or vehicle tocopherol levels. Our interpretation of their studies is that γ-tocopherol in the soy oil antagonized the function of the α-tocopherol that was administered. In a report by Okamoto et al. (66), mice were fed α-tocopherol starting 2 wk before sensitization with OVA, but the form and purity of α-tocopherol were not indicated. The form and purity are important because it has been reported that δ-α-tocopherol vs α-tocopherol succinate can have different outcomes on cell functions (67). Nevertheless, they demonstrated that with α-tocopherol treatment, the number of eosinophils in the BAL was reduced, that IL-4 and IL-5 was reduced, but that IgE was not reduced (66). Differences in these functional effects on cytokines compared with our data may be forms of tocopherols or time of tocopherol administration because they administered tocopherol before sensitization. We acknowledge that our studies focused on determining whether after sensitization, tocopherols could modulate the challenge phase. This is important because patients are already sensitized. In another report, γ-tocopherol in tocopherol-striped corn oil was
administered daily by gavage to rats 2 wk after one OVA sensitization (68). They report a reduced number of eosinophils and lymphocytes in the BAL of the γ-tocopherol-treated mice after two OVA challenges (68). However, the purity of the γ-tocopherol in the corn oil was not reported. Furthermore, the leukocyte infiltration in the OVA response in these rats was predominantly neutrophils rather than the expected predominant eosinophil infiltration (68). In agreement with our studies, they report that tocopherols did not alter lung IL-4 and IL-5 expression (68). In a study examining γ-tocopherol modulation of lung ozone exposure after OVA challenge, control rats that did not receive ozone, but received γ-tocopherol for 4 days beginning after the last OVA challenge, had reduced eosinophils at day 4 after OVA challenge (69). However, because it takes a few days to raise tissue tocopherol levels, which in this protocol is after the peak of eosinophil infiltration, the effect on eosinophils on day 4 after the last OVA challenge was during the resolution phase of eosinophil inflammation. It has also been reported that mice deficient in liver α-TTP exhibit severe deficiency in tissue α- and γ-tocopherol as well as reduced IgE and reduced IL-5 in experimental asthma (70). In these mice, it is not known whether severe tocopherol deficiency during muscle development alters leukocyte hematopoiesis or leukocyte responsiveness. In summary, differences among the reports of tocopherol regulation of experimental asthma most likely reflect differences in the forms of tocopherols, tocopherol concentrations, and time of administration of tocopherols in these studies.

Reports conflict as to whether tocopherols modulate mediators of inflammation such as PGs, cytokines, chemokines, and adhesion molecules. Reports indicate that tocopherols either inhibit or do not inhibit PGE2 synthesis (37, 39, 67, 71–73). It has been reported that α-tocopherol inhibits phorbol ester or endotoxin-induced PGE2 synthesis in vitro in macrophages, endothelial cells, and microglia (37, 39, 74). In contrast, other investigators report that α-tocopherol does not inhibit endotoxin-induced PGE2 synthesis in macrophages, endothelial cells, and epithelial cells (67, 71, 75, 76). Still other reports indicate that γ-tocopherol, but not α-tocopherol, inhibits PGE2 synthesis in Caco2 cells, epithelial cells, and macrophages (72, 77). It has been reported that α-tocopherol and γ-tocopherol inhibit activity of purified cyclooxygenase 2 (36). In in vitro studies of endotoxin-induced inflammation or carrageenan-induced skin air pouch inflammation, γ-tocopherol reduces PGE2 synthesis (73, 78). Our data indicate that purified natural D-α-tocopherol and purified D-γ-tocopherol do not inhibit PGE2 synthesis in vivo in experimental asthma. Whether α-tocopherol modulates endothelial cell adhesion molecule expression in vitro also varies in the literature (35, 79, 80). Briefly, in vitro, α-tocopherol blocks TNF-α and IL-1 induction of VCAM-1 expression by endothelial cells (35, 79–82). In vitro, α-tocopherol is reported to block IL-1β-induced ICAM-1 expression on human aortic endothelial cells, but not on HUVECs, and then in another report, α-tocopherol also does not inhibit TNF-α-stimulated ICAM-1 expression on HUVECs (35, 79). Our data indicate that purified natural D-α-tocopherol and purified D-γ-tocopherol do not alter VCAM-1 expression in vivo in experimental asthma. With regard to cytokines, reports vary as to whether tocopherols have an effect on cytokine expression in vitro and in vivo in animal models of asthma and atherosclerosis (61, 66, 67, 83). In vitro.

With regard to α-tocopherol modulation of cytokines in vivo, reports vary as to whether α-tocopherol has an effect on cytokine expression in animal models of asthma and atherosclerosis (61, 66, 67, 83, 84). With regard to chemokine production, Meydani and colleagues (35) have reported that α-tocopherol does not inhibit spontaneous production of the chemokine MCP-1 by endothelial cells in vitro. Our studies demonstrate that purified natural D-α-tocopherol and D-γ-tocopherol do not alter expression of IL-4, IL-5, IFN-γ, IL-2, eotaxin, MCP-1, or VCAM-1 in the lung. In addition, our data demonstrating that α-tocopherol inhibits leukocyte migration in vitro is consistent with previous reports that α-tocopherol blocks monocyte migration in vitro stimulated by oxidized low density lipoprotein or MCP-1 (40, 85). We suggest that variations in reports on outcomes of tocopherol treatments in vitro and in vivo result, at least in part, from differences in experimental systems, in isoforms and purity of tocopherols, and in concentrations of the tocopherols within different cells. This is important considering our data indicating that forms of tocopherols have opposing regulatory functions on leukocyte recruitment in vivo and in vitro.

Several reports on inhibition of endothelial cell function in inflammation demonstrate that leukocyte trafficking into tissue is inhibited without changes in expression of cytokines, chemokines, or adhesion molecules. It has been reported that in chimeric gp91phox-deficient mice, in chimeric Go2i-deficient mice, and in mice treated with the antioxidant bilirubin, leukocyte recruitment is blocked in experimental asthma without altering Ag-stimulated expression of the adhesion molecule VCAM-1 and without altering Ag-stimulated lung lavage levels of the cytokines IL-2, IFN-γ, IL-4, IL-5, IL-6, IL-10, TNF-α, and IL-12, or the chemokines MCP-1 and eotaxin (1, 46, 47). Consistent with this, our data indicate that α-tocopherol and γ-tocopherol regulate endothelial cell function during experimental asthma without altering expression of cytokines, chemokines, or adhesion molecules in vivo or in vitro.

In summary, we identified novel opposing regulatory functions for the two major forms of vitamin E during asthma. Furthermore, these opposing regulatory functions are consistent with the disparate outcomes of vitamin E on asthma in studies of Americans vs Europeans and with the disparate outcomes of vitamin E on asthma in animal studies. We demonstrated that a mechanism for opposing immunoregulatory functions of D-α-tocopherol and D-γ-tocopherol during experimental asthma is, at least in part, by direct regulation of endothelial cell signals. The levels of tocopherols in this report may have general relevance to regulation of leukocyte recruitment in inflammation because there is a direct regulatory function of tocopherols in the endothelium during leukocyte recruitment and because endothelial cell function regulates leukocyte recruitment in several types of inflammation. Furthermore, information about differential tocopherol regulation of inflammation will provide a basis toward designing drugs and diets that more effectively modulate these pathways and improve health. Information from our studies will have significant impact on interpretation of vitamin E clinical studies, on the design of future clinical studies with vitamin E, and on our understanding of vitamin E regulation of vascular function during leukocyte recruitment.

Disclosures

The authors have no financial conflict of interest.

References


Corrections


In Fig. 6D, the $\alpha$T (80$\mu$M) and $\gamma$T (5$\mu$M) labels on the $x$-axis were switched. The corrected figure is shown below.