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ISOFORMS OF VITAMIN E DIFFERENTIALLY REGULATE INFLAMMATION

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Abstract

Vitamin E regulation of disease has been extensively studied in humans, animal models and cell systems. Most of these studies focus on the α -tocopherol isoform of vitamin E. These reports indicate contradictory outcomes for anti-inflammatory functions of the α -tocopherol isoform of vitamin E, especially with regards to clinical studies of asthma and atherosclerosis. These seemingly disparate clinical results are consistent with recently reported unrecognized properties of isoforms of vitamin E. Recently, it has been reported that physiological levels of purified natural forms of vitamin E have opposing regulatory functions during inflammation. These opposing regulatory functions by physiological levels of vitamin E isoforms impact interpretations of previous studies on vitamin E. Moreover, additional recent studies also indicate that the effects of vitamin E isoforms on inflammation are only partially reversible using physiological levels of a vitamin E isoform with opposing immunoregulatory function. Thus, this further influences interpretations of previous studies with vitamin E in which there was inflammation and substantial vitamin E isoforms present before the initiation of the study. In summary, this review will discuss regulation of inflammation by vitamin E, including alternative interpretations of previous studies in the literature with regards to vitamin E isoforms.

Keywords

allergy; atherosclerosis; inflammation; reactive oxygen species; tocopherol; transendothelial migration; VCAM-1; vitamin E

Introduction

Vitamin E is commonly used as an antioxidant to try to limit oxidative damage and inflammatory disease. Vitamin E is an antioxidant lipid vitamin that consists of multiple natural and synthetic forms. The natural forms of vitamin E include α -tocopherol, β -tocopherol, γ -tocopherol, and δ -tocopherol as well as the tocotrienol forms of each of these [1, 2] (Figure 1). Plants synthesize the lipids tocopherols and tocotrienols from tyrosine and chlorophyll [3, 4]. Then, these tocols are consumed in the diet from plant lipids. Tocols are loaded in intestinal-formed chylomicrons. These chylomicrons are transported in the lymph to the lymph nodes and to the liver, where the tocols are transferred to lipid particles. α -tocopherol and γ -tocopherol isoforms are the most abundant in diets, supplements, and tissues. However, the α -tocopherol isoform is about 10 fold higher than γ -tocopherol in tissues since there is preferential transfer of the α -tocopherol isoform to lipid particles by the liver α -tocopherol transfer protein [5]. At equal molar concentrations in vitro, it is

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reported that the α -tocopherol and γ -tocopherol isoforms and the tocotrienol forms have relatively similar capacity to scavenge reactive oxygen species (ROS) during lipid oxidation [1, 6, 7]. Thus, in vivo, there is likely more ROS scavenging by α -tocopherol than γ -tocopherol because it is at a 10 fold higher concentration in the tissues. Moreover, the antioxidant capacity of the tocopherols is influenced by membrane uptake of the tocopherols and by tocopherol mobility and localization in membranes [6, 7]. In addition to scavenging ROS, γ -tocopherol, in contrast to α -tocopherol, also reacts with nitrogen species such as peroxynitrite forming 5-nitro- γ -tocopherol [8-10]. This 5-nitro- γ -tocopherol can react with hypochlorous acid to form chlorinated tocopherols or quinones [10, 11]. The chlorinated tocopherols and quinones may be formed during leukocyte generation of hypochlorous acid during inflammation. The oxidized tocopherols are recycled by reduction by vitamin C [12-14]. Without reduction of vitamin E by vitamin C, vitamin E can act as ROS donor [15]. In mice, vitamin C is endogenously synthesized whereas humans must consume vitamin C [16]. Thus, some clinical studies have supplemented patients with both tocopherols and vitamin C [17-20]. In vivo, tocopherols are metabolized to carboxyethyl-hydroxychromans (CEHC) and excreted [2, 21]. The CEHC forms are reported to also have regulatory functions [22]. Physiological plasma levels of α -CEHC and γ -CEHC are in the nanomolar range whereas γ - and α -tocopherols are 2-25 μ M in the plasma [23, 24]. Importantly, besides the antioxidant capacity of the tocopherols, it has been reported that tocopherols also have non-antioxidant functions [1, 25, 26]. In summary, as antioxidants, tocopherols regulate reactive-oxygen-species cell signaling and oxidative damage; the non-antioxidant functions of tocopherols also regulate cell signaling. Thus, the focus of this review is the antioxidant and non-antioxidant functions of tocopherol isoforms during inflammation.

During inflammation, cytokines induce adhesion molecule expression on the endothelium (Figure 2). Leukocytes bind to these adhesion molecules and then the bound leukocytes are recruited into tissues by chemokines/chemoattractants (Figure 2). The specificity of leukocyte homing to tissues is regulated by the combination of chemokines in the microenvironment, adhesion molecules on the endothelium and leukocyte receptors for these chemokines and adhesion molecules [27]. Furthermore, the combination of vascular adhesion molecules expressed by an endothelial cell is dependent on the stimulant(s) for endothelial activation [28]. The expression of adhesion molecules is induced by several mediators including high levels of reactive oxygen species, cytokines produced in the tissue, turbulent vascular fluid shear stress, or microbial stimulation of endothelial toll-like receptors [29-36]. Thus, the microenvironment stimuli regulate the specificity of leukocyte recruitment. The binding of leukocytes to the endothelium and the specificity of these interactions have been discussed in previous reviews [37-46].

Leukocyte binding to adhesion molecules activates signal transduction within the endothelial cells that then induces the opening of narrow vascular passageways through which leukocytes migrate (Figure 2) [46-48]. Leukocyte movement through these passageways is stimulated by chemokines that are produced by the endothelium and the tissue (Figure 2). If adhesion molecule signal transduction is inhibited, leukocytes bind to the endothelium but do not complete transendothelial migration [49]. The cells that bind to the endothelium but do not complete transendothelial migration often release from the endothelium and continue in the blood flow as demonstrated by intravital microscopy. Thus, the endothelial cell adhesion molecules and their intracellular signals are a source for intervention in leukocyte recruitment during inflammation. One of these adhesion molecules, vascular cell adhesion molecule-1 (VCAM-1), signals through low levels of ROS [47, 50, 51]. Moreover, this VCAM-1-dependent signaling through low levels of ROS is regulated by tocopherols [26]. After granulocytes and monocytes enter tissues, they release high levels of reactive oxygen species to destroy pathogens and as a consequence cause

some tissue damage. These high levels of reactive oxygen species are also a target for regulation by tocopherols.

The endothelial cell and leukocyte production of ROS is catalyzed by several enzymes including NADPH oxidase, nitric oxide synthase, cyclooxygenase, and xanthine oxidase. NADPH oxidase catalyzes the conversion of oxygen to superoxide that then dismutates either spontaneously to hydrogen peroxide or is converted to hydrogen peroxide by superoxide dismutase. Hydrogen peroxide is converted to water and oxygen by catalase or it is metabolized to reactive hydroxyl radicals by the Haber Weiss Reaction [52]. Furthermore, hydrogen peroxide can react with nitric-oxide-synthase-generated nitric oxide to produce peroxynitrate [52]. Furthermore, these ROS can oxidize lipids that then act as donors of reactive oxygen species. Thus, it is critical to maintain a balance of reactive oxygen species generation in vivo. To balance oxidation, cells have antioxidant mechanisms, such as superoxide dismutase, catalase, glutathiones, peroxiredoxins, and antioxidant vitamins E and C.

VCAM-1 function in disease and infections

Vitamin E has been used to try to regulate inflammatory diseases that involve VCAM-1-mediated leukocyte recruitment such as asthma, arthritis and atherosclerosis. VCAM-1 signal transduction occurs through ROS and protein kinase C, both of which are regulated by vitamin E. However, there are contradictory outcomes for vitamin E in clinical studies of asthma and atherosclerosis. In addition, there are contradictory outcomes for vitamin E supplementation in animal models of inflammation. These clinical and experimental studies have primarily focused on analysis of one form of vitamin E, α -tocopherol, even though multiple forms of vitamin E are present in the studies. Our recent report on novel properties of tocopherol isoforms [26] suggests that the reported contradictory outcomes of the previous studies is consistent with unrecognized functions for the combination of vitamin E isoforms that were present in these previous studies.

VCAM-1, which signals through ROS, has a regulatory role in peripheral tissue inflammation in several diseases. In these diseases, there are different leukocyte cell types that bind to VCAM-1 via the leukocyte ligand $\alpha 4\beta 1$ -integrin. This is, at least in part, a result of leukocyte specific chemokine activation of $\alpha 4\beta 1$ -integrin in its integrin high affinity conformation [37, 43-45]. In allergic disease, blocking VCAM-1 by intravenous injection of anti-VCAM-1 blocking antibodies inhibits eosinophil recruitment in asthma models in several species [53-55]. Furthermore, in allergic disease, blocking VCAM-1 or using VCAM-1 knockout mice inhibits mast cell precursor binding to endothelium and inhibits recruitment of mast cell precursors to antigen-stimulated lungs and intestine [56-60]. In a mouse model of atopic dermatitis, VCAM-1 blockade reduces severity of inflammatory disease and delays the onset of disease [61]. In inflammatory bowel disease, antibody inhibition of VCAM-1 blocks T cell infiltration into the intestine [62]. In an experimental model of multiple sclerosis, blocking VCAM-1 inhibits T cell infiltration into the brain [63]. Consistent with this, multiple sclerosis patients have elevated VCAM-1 but not MAdCAM-1 expression in brain tissue [64]. In clinical trials, blocking the VCAM-1 ligand, $\alpha 4$ -integrin, with antibodies (natalizumab) reduces disease severity in multiple sclerosis and Crohn's disease [65, 66]. VCAM-1 also has a role in regulation of infection because during lymphocytic choriomeningitis virus (LCMV) infections, VCAM-1 on the endothelium mediates CD8+ T cell, monocyte and dendritic cell infiltration into the brain [67]. In experimental visceral leishmaniasis, VCAM-1 interaction with $\alpha 4\beta 1$ integrin regulates the production of dendritic cells since antibody inhibition of VCAM-1 or $\alpha 4\beta 1$ -integrin blocks the dendritic cell response in the spleens in these mice [68]. In summary, VCAM-1 functions in allergic and infection-induced inflammation.

It has also been reported that VCAM-1 has an important role in cardiovascular diseases and in the embryonic development of the cardiovascular system. VCAM-1 is required for development of the heart since the VCAM-1 knockout mouse is an embryonic lethal due to malformation of the heart [69]. In atherosclerosis, VCAM-1 is the first adhesion molecule expressed prior to atherosclerotic plaque development [70]. In the carotid artery, neointimal formation is reduced by VCAM-1 siRNA or by antibody blockade of the VCAM-1 ligand $\alpha 4\beta 1$ -integrin in rodents [71, 72]. VCAM-1 is also linked to calcification of aortic stenosis in patients with coronary artery disease [73]. In the atherosclerotic carotid arteries, VCAM-1 mediates monocyte adhesion as demonstrated with anti-VCAM-1 blocking antibodies [74]. In cardiac allografts, lower levels of VCAM-1 expression are indicative of a reduction in rejection [75, 76]. In ischemia-reperfusion of the liver, blocking VCAM-1 inhibits leukocyte recruitment and injury [77]. Thus, VCAM-1, which signals through ROS, has an important regulatory role in cardiovascular diseases.

During inflammation, VCAM-1 expression is induced on endothelial cells. VCAM-1 is expressed on the luminal surface and the lateral surface but not the basal surface of endothelial cells. At these sites, VCAM-1 activates intracellular signals in endothelial cells (Figure 2, 3). The signals in this pathway are transient and occur within minutes, consistent with the transient, rapid nature of leukocyte transendothelial migration. Activation of VCAM-1 stimulates calcium channels, intracellular calcium release, and the small molecular weight G protein Rac1 for the activation of the NADPH oxidase NOX2 [47, 78]. VCAM-1 does not activate other enzymes that generate reactive oxygen species [47]. The activated NOX2 generates superoxide that then dismutates to hydrogen peroxide (H_2O_2), generating $1\ \mu M\ H_2O_2$ during VCAM-1 signaling [78, 79]. This $1\ \mu M\ H_2O_2$ is relatively low as compared to the $50\text{--}200\ \mu M\ H_2O_2$ produced by macrophages or neutrophils in tissues [80, 81]. It is also much lower than the exogenous $100\text{--}1000\ \mu M\ H_2O_2$ used in studies on oxidative damage [82–86]. These differences in H_2O_2 levels are important in understanding functions of oxidation as we and others reported that $1\ \mu M\ H_2O_2$ and $>50\ \mu M\ H_2O_2$ have opposing effects on signal transduction [50, 51, 87, 88].

During VCAM-1 signaling, the $1\ \mu M\ H_2O_2$ oxidizes the pro-domain of matrix metalloproteinases (MMPs), causing autocatalytic cleavage of the pro-domain and activation of endothelial cell-associated MMPs within minutes [51] (Figure 3). In contrast, there is a several hour delay for degradation of leukocyte-associated tissue inhibitors of metalloproteinases (TIMPs) and thus activation of leukocyte MMPs by VCAM-1-induced endothelial-derived H_2O_2 [51] (Figure 3). Moreover, the activation of endothelial cell-associated MMPs but not the leukocyte MMPs is required for VCAM-1-dependent leukocyte migration which occurs in minutes [51]. During VCAM-1 signaling, the H_2O_2 also diffuses through cell membranes at $100\ \mu m/second$ [89]. In contrast, superoxide has a relatively low diffusion rate across membranes [89]. During VCAM-1 signal transduction, the $1\ \mu M\ H_2O_2$ directly oxidizes and transiently activates intracellular protein kinase C α (PKC α) in endothelial cells [50]. This VCAM-1-induced oxidative-activation of PKC α then induces phosphorylation and activation of protein tyrosine phosphatase 1B (PTP1B) [90]. Interestingly, the PTP1B which has an oxidizable cysteine in its catalytic domain is not oxidized during VCAM-1 signaling in endothelial cells [90], indicating specificity of targets for oxidation by the low concentrations of reactive oxygen species generated during VCAM-1 signaling. The signals downstream of the PTP1B that regulate endothelial cell junctions are currently under further investigation. In addition, during VCAM-1 signaling for leukocyte transendothelial migration, there is activation of the G protein G $\alpha i 2$ [91]. Importantly, the signals in Figure 3 have been demonstrated to function in regulation of VCAM-1-dependent leukocyte transendothelial migration in vitro and in vivo [26, 47, 49–51, 78, 90–92]. In summary, VCAM-1 functions as a scaffold for leukocyte adhesion and functions in the activation of ‘outside-in’ ROS signal transduction in endothelial cells.

Vitamin E isoform-specific regulation of VCAM-1 function in vitro and in vivo

We reported that, in vitro, natural d- α -tocopherol blocks whereas natural d- γ -tocopherol elevates VCAM-1-dependent lymphocyte transmigration at physiological concentrations [26]. Moreover, treatment with γ -tocopherol ablates the inhibition by α -tocopherol such that the lymphocyte transmigration is the same as the vehicle-treated control [26]. Interestingly, this occurs at physiological tocopherol concentrations. Thus, γ -tocopherol ablates the effects of α -tocopherol, even though, in tissues, γ -tocopherol is at 1/10 the concentration of α -tocopherol [26]. These regulatory functions of the tocopherols on lymphocyte transmigration are through a direct effect of the tocopherols on endothelial cells because pretreatment of the endothelial cells with α -tocopherol or γ -tocopherol overnight inhibits and elevates, respectively, lymphocyte transmigration in vitro [26]. In contrast, pretreatment of the lymphocytes with physiological concentrations tocopherols has no effect on VCAM-1-dependent lymphocyte transmigration [26]. The γ -tocopherol-induced elevation of transendothelial migration is VCAM-1-dependent since anti-VCAM-1 blocking antibodies inhibit lymphocyte transmigration [26]. Although the tocopherols regulate VCAM-1-dependent leukocyte transendothelial migration, the tocopherols do not modulate lymphocyte binding to VCAM-1 on the endothelial cells [26]. It has also been reported that pretreatment of endothelial cells with α -tocopherol and washing of aortic endothelial cells blocks monocyte migration in vitro [93]. In another report, α -tocopherol blocks transendothelial migration of monocytes stimulated by oxidized-LDL or the chemokine MCP-1 [94]. However, these reports on α -tocopherol modulation of monocyte migration did not address the adhesion molecules involved in the responses and did not examine whether γ -tocopherol regulated endothelial cell or leukocyte function during transmigration. We reported that the tocopherols at physiological levels modulate endothelial function during VCAM-1-dependent transmigration by altering VCAM-1-induced oxidative activation of endothelial cell PKC α (Figure 4) [26]. Specifically, the VCAM-1-induced activation of PKC α is inhibited by α -tocopherol and this inhibition of PKC α by α -tocopherol is ablated by γ -tocopherol [26]. It is also reported by Martin-Nizard that thrombin-stimulated activation of endothelial cell PKC is inhibited by α -tocopherol [95]. In summary, the α -tocopherol and γ -tocopherol have opposing regulatory functions on VCAM-1 signaling during leukocyte transmigration in vitro.

We reported that in vivo, supplemental doses of α -tocopherol and γ -tocopherol, that result in physiological tissue levels of these tocopherols, have opposing regulatory functions on recruitment of leukocytes to the lung during allergic inflammation [26]. In a model of allergic inflammation, animals are sensitized by intraperitoneal administration of chicken egg ovalbumin (OVA) in adjuvant and then the lung is challenged with OVA in saline [26]. During allergic inflammation in the lung, eosinophil migration is dependent on VCAM-1 whereas the other leukocytes can migrate on the adhesion molecule ICAM-1 (Figure 5) [54, 55]. However, both VCAM-1 and ICAM-1 signal through PKC α [50, 96] and it is reported that PKC α can be regulated by tocopherols [97, 98]. In our report, we focused on supplementation with tocopherols after OVA antigen sensitization to determine whether tocopherols modulate the OVA antigen-challenge phase (Figure 6) [26]. This is important because patients are already sensitized. Supplementation with tocopherols after OVA-sensitization and during OVA-challenge raised tissue tocopherols 5-7 fold higher than mice consuming control rodent chow; this does not affect body weight or lung weight [26, 99]. Consistent with the in vitro studies with tocopherol regulation of leukocyte migration, physiological levels of d- γ -tocopherol elevates leukocyte accumulation in the bronchoalveolar lavage and lung tissue in response to OVA challenge (Figure 6) [26]. In contrast, physiological levels of d- α -tocopherol inhibit OVA-induced lung inflammation

(Figure 6). Moreover, in vivo, this physiological level of d- γ -tocopherol, at only 10% the tissue concentration of d- α -tocopherol, ablates the anti-inflammatory benefit of the d- α -tocopherol isoform in response to OVA challenge (Figure 6) [26]. Furthermore, the levels of tocopherols in this study do not alter numbers of blood eosinophils, indicating that eosinophils were available for recruitment. The opposing functions of purified d- α -tocopherol or d- γ -tocopherol in vivo is also not through modulation of expression of several cytokines, chemokines, prostaglandin E₂, or vascular adhesion molecules which regulate inflammation because these were not altered by tocopherol supplementation [26]. This modulation of leukocyte infiltration in allergic inflammation, without alteration of adhesion molecules, cytokines or chemokines, is similar to several previous reports of in vivo inhibition of lung inflammation by inhibition of intracellular signals in endothelial cells [49, 91, 92]. In summary, physiological levels of α -tocopherol and γ -tocopherol have opposing regulatory function on allergic inflammation that is, at least in part, by regulation of VCAM-1 activation of PKC α [26]. The opposing functions of tocopherol isoforms have important implications for the interpretation of clinical studies and animal studies of vitamin E regulation of inflammation.

In our report that supplemental doses of the α -tocopherol and γ -tocopherol isoforms of vitamin E decrease and increase, respectively, lung inflammation [26], the supplemental doses of tocopherol raised plasma tocopherol 5 fold [26]. However, in this previous study, the reported 2mg/day dose for tocopherols were suspended for only a couple of minutes before administration to the animals [26]. We have recently found that with only a couple of minutes of suspension time, the final tocopherol suspension is actually at a 0.2 mg/day dose because complete suspension of tocopherols in the vehicle ethoxylated castor oil requires at least 20 minutes as determined by HPLC (McCary et al, manuscript submitted). Moreover, we recently reported that the completely suspended 0.2 mg tocopherol/day (as determined by HPLC) raises the plasma tocopherol 5 fold and is comparable to the 5 fold increase in plasma tocopherol in our previous report [26]. Furthermore, the 0.2 mg dose of tocopherols subcutaneously administered daily during OVA challenge demonstrated the anti-inflammatory and pro-inflammatory regulatory functions of α -tocopherol and γ -tocopherol, respectively (Figure 6) (McCary et al, manuscript submitted) as in our previous report [26]. In summary, we define completely suspended 0.2 mg tocopherol subcutaneous treatment/day as “supplemental tocopherol treatment” since it raises plasma tocopherols 5 fold. Importantly, this information on suspension of tocopherol affects interpretations of reports on vitamin E in which tocopherols were suspended in oil vehicles because incomplete suspension can result in different dose-dependent experimental outcomes. Differential dose-dependent outcomes for tocopherols are discussed in the next section.

In summary, natural d- α -tocopherol and natural d- γ -tocopherol differ in structure by only one methyl group (Figure 1) but, at physiological tissue concentrations, these tocopherols have opposing regulatory effects on leukocyte recruitment and VCAM-1 signal transduction [26].

Reversibility of tocopherol isoform-specific regulation of inflammation

We demonstrated that changing the isoform of tocopherol only partially reverses the effects of supplemental α -tocopherol or γ -tocopherol treatment in the OVA-challenge model (Figure 6) (McCary et al, manuscript submitted). In these studies, mice were sensitized by intraperitoneal injection of OVA in the adjuvant alum. Then in phase 1 of the study, supplemental levels of α -tocopherol or γ -tocopherol were administered beginning after sensitization, and during the 3 intranasal challenges with OVA in saline (Figure 6A). Following phase 1, there was a 4-week clearance phase during which mice were not treated with tocopherols or challenged with OVA (Figure 6A). Four weeks is sufficient time to clear

supplemented tocopherol from plasma and lung tissue and to clear inflammation from the lung [100, 101] (McCary et al, manuscript submitted). After the clearance phase, mice entered phase 2 of treatment during which they received supplemental α -tocopherol or γ -tocopherol and were challenged with OVA (Figure 6A). Treatment with supplemental α -tocopherol in both phases reduced OVA-induced inflammation and treatment with γ -tocopherol in both phases raised OVA-induced inflammation (Figure 6B)(McCary et al, manuscript submitted). For OVA-challenged mice that received supplemental γ -tocopherol in phase 1 and then supplemental α -tocopherol in phase 2, there was not a complete reduction in inflammation to the level of the mice that had α -tocopherol treatment in both phases (Figure 6B). Instead, this switch from γ -tocopherol in phase 1 to α -tocopherol in phase 2 resulted in an intermediate phenotype with OVA-induced inflammation similar to the level of vehicle-treated OVA-challenged mice (Figure 6B). An intermediate phenotype also occurred for OVA-challenged mice treated with α -tocopherol in phase 1 followed by γ -tocopherol in phase 2 (Figure 6B). Thus, switching the tocopherol form in phase 2 did not completely reverse the OVA-induced inflammatory phenotype to the level of inflammation when the same tocopherol isoform was present in both phases. This has important implications for clinical studies where dietary tocopherols influence the outcomes of tocopherol supplementation.

Interestingly, increasing α -tocopherol 10 fold above supplemental levels in phase 2 completely inhibits the elevated OVA-induced inflammation in the lung lavage of animals that received supplemental γ -tocopherol in phase 1 (Figure 6B) (McCary et al., manuscript submitted). In contrast, a 5 fold increase in α -tocopherol was not sufficient to inhibit the enhanced inflammation in the lung lavage induced by supplemental γ -tocopherol (McCary et al, manuscript submitted). The subcutaneous administration of the 10 fold levels of α -tocopherol raised plasma α -tocopherol 50 fold (McCary et al., manuscript submitted). Thus, we define this 10 fold higher level of tocopherol as highly-elevated tocopherol. In summary, highly elevated α -tocopherol reversed the pro-inflammatory effects of supplemental levels of γ -tocopherol on inflammation in the lung lavage.

Since, at supplemental levels of tocopherols, γ -tocopherol is in tissues at 1/10 the concentration of α -tocopherol [26], we determined whether raising tissue γ -tocopherol to equal molar tissue concentrations of supplemental α -tocopherol yielded the same anti-inflammatory functional outcome as the α -tocopherol. To raise the tissue concentrations of γ -tocopherol, a 10 fold higher (highly-elevated) level of γ -tocopherol than supplemental γ -tocopherol was administered subcutaneously (Figure 6B). This yielded 30 μ M plasma γ -tocopherol. We found that when γ -tocopherol was loaded in tissues to the same concentration as supplemental levels of α -tocopherol, γ -tocopherol was anti-inflammatory. Furthermore, when examining reversibility of the effects of highly-elevated γ -tocopherol, highly elevated doses of γ -tocopherol in phase 1 and/or phase 2 blocked lung lavage inflammation (Figure 6B) (McCary et al, manuscript submitted). In addition, when highly-elevated doses of γ -tocopherol were present in phase 1 but no tocopherol was administered in phase 2, the inhibitory effects of γ -tocopherol on lung lavage inflammation were ablated (Figure 6B) (McCary et al, manuscript submitted). Thus, at equal molar tissue levels of α -tocopherol and γ -tocopherol, these tocopherols both function to inhibit OVA-induced lung lavage inflammation. However, in lung tissue (data not shown), highly-elevated levels of γ -tocopherol increased tissue inflammation and this was reversible (McCary et al, manuscript submitted).

It is important to note that the highly-elevated γ -tocopherol levels achieved by subcutaneous tocopherol treatments are likely not achievable by dietary means. In a report by Bella et al [102], mice fed a diet containing 1150 mg α -tocopherol acetate per kg diet for 15 days achieved approximately 13 μ g/ml α -tocopherol in the plasma. If dietary γ -tocopherol had

been administered at this dose, the plasma level of γ -tocopherol would be less than 13 $\mu\text{g}/\text{ml}$ because of the 10 fold lower efficiency for γ -tocopherol incorporation into lipid particles by α -TTP in the liver. In our study, subcutaneously administered highly-elevated tocopherol treatment (2 mg/day for 8 days) is calculated as approximately equivalent to about a 500 mg tocopherol per kg diet when adjusting for mouse diet consumption per day. However, in the study by McCary et al (manuscript submitted) which examined equal molar functionality of the tocopherols, subcutaneously administered highly-elevated γ -tocopherol treatment (2 mg/day for 8 days) resulted in 25 $\mu\text{g}/\text{ml}$ γ -tocopherol in plasma. In addition, subcutaneously administered highly-elevated α -tocopherol treatment (2 mg/day for 8 days) resulted in 75 $\mu\text{g}/\text{ml}$ α -tocopherol in plasma. Thus, the highly-elevated tocopherol tissue levels that were achieved through subcutaneous administration would not likely have been achieved through dietary means.

In summary, pro-inflammatory supplemental γ -tocopherol at physiological tissue levels functions differently than the anti-inflammatory physiological levels of α -tocopherol. In addition, at equal-molar tissue levels, α -tocopherol and γ -tocopherol function similarly to inhibit inflammation in the lung lavage. Furthermore, since OVA-induced inflammation in the lung lavage is elevated by supplemental physiological levels of γ -tocopherol but inhibited by highly-elevated levels of γ -tocopherol, it suggests that at supplemental levels, γ -tocopherol may have non-antioxidant pro-inflammatory functions. These results have implications for clinical studies with tocopherols and alter interpretations of tocopherol studies reported in the literature.

Importantly, although high levels of tocopherols have been used clinically, they can have adverse effects on the brain in animals and humans. In rats, very high doses of α -tocopherol (2600mg α -tocopherol/day) increase blood pressure and adversely affect the central nervous morphology as demonstrated by changes in brain microglia, Purkinje cells, and glial filaments [103]. This is consistent with observations of high blood pressure observed in humans with α -tocopherol supplementation [104]. In addition, several reports indicate that high dose vitamin E may increase hemorrhagic stroke, all-cause mortality, or post trial cerebral infarction [105-109]. Yet, in a few other studies, vitamin E did not increase the incidence of stroke [110]. Whether the combination of tocopherol isoforms or levels of tocopherol isoforms influence these adverse outcomes is not known. In summary, supplemental levels of α -tocopherol and γ -tocopherol have opposing effects on inflammation, whereas elevating tocopherols further may result in additional concerns regarding adverse effects on the brain.

Other antioxidants also regulate VCAM-1 signals through ROS in vitro and in vivo

VCAM-1 signal transduction, which occurs through ROS activation of matrix metalloproteinases (MMPs), is also regulated by the antioxidant bilirubin, supporting the importance of redox regulation of this signaling pathway in VCAM-1-dependent leukocyte recruitment during inflammation [92]. Bilirubin is generated from heme by hemoxygenase-1 [92, 111]. After generation of bilirubin, it can undergo redox cycling such that oxidation of bilirubin converts it to biliverdin [111-114]. Bilirubin and biliverdin are membrane permeable [114, 115]. Biliverdin is recycled back to bilirubin by biliverdin reductase and the cofactor NADPH [92]. Hemoxygenase-1 and biliverdin reductase are expressed by endothelial cells [92]. Furthermore, bilirubin is taken up by endothelial cells and thus bilirubin as well as tocopherols in endothelial cells can function as antioxidants [92]. It has also been reported that bilirubin acts as an antioxidant in that it reduces oxidized phospholipids in vitro with the approximate rate of antioxidant vitamins [116].

In vitro studies demonstrate that preloading endothelial cells with the antioxidant bilirubin blocks VCAM-1-dependent lymphocyte transendothelial migration and blocks VCAM-1 activation of MMPs [92]. These concentrations of bilirubin in the upper physiological range do not affect cell viability [92]. Consistent with an antioxidant function for bilirubin, VCAM-1-dependent lymphocyte migration is not blocked by the stable bilirubin conjugate ditaurobilirubin which cannot scavenge ROS [92]. The bilirubin inhibition of lymphocyte migration results from an inhibition of migration rather than inhibition of lymphocytes available for migration as the number of lymphocytes bound to the endothelial cell monolayer is unaffected by bilirubin [92]. Therefore, the antioxidant bilirubin blocks VCAM-1-dependent lymphocyte migration across endothelial cells by, at least, blocking the ROS-mediated activation of MMPs.

Consistent with the inhibitory function of bilirubin on VCAM-1-dependent leukocyte transmigration in vitro, bilirubin blocks VCAM-1-dependent leukocyte migration in vivo [92]. This was examined in OVA-induced VCAM-1-dependent lung inflammation (Figure 5) [92]. In studies examining bilirubin regulation of recruitment of eosinophils during allergic inflammation, mice were sensitized by intraperitoneal injection of OVA in the adjuvant alum and then challenged by intranasal inhalation of OVA [92]. At the time of intranasal OVA challenge, mice also received either intraperitoneal injections of bilirubin at upper physiological concentrations or vehicle control [92]. The treatment with bilirubin inhibits eosinophil infiltration into the bronchoalveolar lavage by >90% and inhibits lymphocyte infiltration by 60% [92]. The migration of eosinophils into the lung tissue is also reduced by 90% by the bilirubin treatments [92]. The reduction in eosinophil and lymphocyte infiltration is consistent with the VCAM-1 dependence of eosinophil migration and the partial VCAM-1 dependence of lymphocyte migration in this lung response to OVA [53-55]. There is no effect of bilirubin on OVA-induced infiltration of monocytes or neutrophils [92]. This lack of effect on monocytes and neutrophils is as expected because recruitment of monocytes and neutrophils in this model is independent of binding to VCAM-1.

After bilirubin treatment, there are sufficient numbers of eosinophils available for migration as there is not a reduction in blood eosinophils in the bilirubin-treated group compared to the nontreated group [92]. In fact, there is a 3 fold increase in blood eosinophil numbers with bilirubin administration which is consistent with inhibition of blood eosinophils transendothelial migration [92]. In addition, VCAM-1 expression in OVA-treated mice was not altered by bilirubin treatment, indicating that VCAM-1 was available for eosinophil binding to the endothelium [92].

Bilirubin treatment also does not alter the OVA-induced increases in Th2 cytokines (IL-4, IL-5, IL-6 or IL-10) in lung lavage fluid or in OVA-restimulated draining lymph node cells [92]. Bilirubin does not alter the OVA-induced increase in the chemokines MCP-1 or eotaxin and it also does not increase expression of Th1 cytokines (IL-2, IL-12, IFN γ or TNF α) which are not expected to be upregulated by OVA stimulation [92]. In summary, VCAM-1-dependent eosinophil and lymphocyte infiltration into the lung is reduced by the antioxidant bilirubin without altering the expression of the VCAM-1, cytokines, or chemokines that regulate eosinophil infiltration in response to OVA. These data are consistent with bilirubin scavenging of endothelial-cell derived ROS generated during VCAM-1 signaling. In summary, bilirubin and α -tocopherol are antioxidants that block VCAM-1 signals through ROS in vitro and inhibit VCAM-1-dependent eosinophilia in allergic responses in mice. Moreover, bilirubin has approximately the same rate at reducing oxidized phospholipids as antioxidant vitamins [116] and it is reported that bilirubin levels are significantly lower in severe asthma in a study from Australia [117], suggesting altered regulation of inflammation in asthmatics by antioxidant vitamins and bilirubin.

New interpretations for reports on tocopherol regulation of inflammation in experimental models

Our data on regulation of inflammation by supplemental and highly-elevated levels of tocopherol isoforms alter interpretations of animal studies with tocopherol modulation of inflammation. Many reports with animal studies indicate that vitamin E was administered to animals but the form, source, and purity of tocopherols are often not reported. Furthermore, the tissue levels of tocopherol isoforms after administration are sometimes not determined. Another source of confounding factors in studies is the lack of consideration for tocopherol isoforms that are present in the oils in animal and human diets or in the oil vehicles used for delivery of the tocopherols. We and others have determined the levels of α -tocopherol and γ -tocopherol in dietary oils (Figure 7) [21, 24, 26]. In rodent studies, rodent chow contains α -tocopherol but low to no γ -tocopherol. However, in some reports for allergic inflammation, α -tocopherol is administered in oil vehicles that contain other tocopherol isoforms. In a report by Suchankova et al [118], purified α -tocopherol was administered in soy oil by gavage and they found no major effect of α -tocopherol on immune parameters or lung airway responsiveness in mice challenged with OVA. However, the soy oil vehicle used in this study should contain an abundance of γ -tocopherol (Figure 7) and they did not measure tissue tocopherol levels or vehicle tocopherol levels. Our interpretation of this study is that γ -tocopherol in the soy oil antagonized the function of the α -tocopherol that was administered. In another report, γ -tocopherol in tocopherol-stripped corn oil was administered daily by gavage to rats two weeks after one OVA sensitization and then the rats received two OVA challenges [119]. In this report, there was a reduced number of eosinophils and lymphocytes in the BAL of the γ -tocopherol-treated mice after OVA challenge [119]. However, the purity of the γ -tocopherol in the corn oil vehicle was not reported. Moreover, in this study, the leukocyte infiltration in the OVA response in these rats was predominantly neutrophils rather than the expected predominant eosinophil infiltration after several OVA challenges [119]. Furthermore, if highly-elevated levels of γ -tocopherol were achieved in the study, this is consistent with our report of inhibition of allergic inflammation with highly elevated doses of γ -tocopherol (McCary et al manuscript submitted). It has also been reported by Okamoto et al [120] that in mice fed α -tocopherol starting 2 weeks before sensitization with OVA, there is a reduction in the number of eosinophils in the bronchoalveolar lavage, a reduction in the cytokines IL-4 and IL-5, and a reduction in airway hyperresponsiveness, even though the form and purity of α -tocopherol were not indicated. In addition, Mabalirajan et al [121] reported that oral administration of 0.4 mg α -tocopherol/mouse/day in ethanol after sensitization blocked OVA-induced lung inflammation and mitochondrial dysfunction [121]. In this report by Mabalirajan et al [121] α -tocopherol-treatment reduced AHR, IL-4, IL-5, IL-13, OVA-specific IgE, eotaxin, TGF β , 12/15-LOX, lipid peroxidation, and lung nitric oxide metabolites, while restoring cytochrome-c oxidase in lung mitochondria and bronchial epithelia [121]. Thus, conflicting reports of tocopherol regulation of OVA-induced inflammation are likely outcomes of differences in isoforms of tocopherols present in the studies from diet, administration, and oil vehicles.

Tocopherols have also been used to scavenge ROS after ozone challenge to the lung. In asthmatic children exposed to ozone, vitamin E and C supplementation reduces IL-6 in nasal lavages, although the form of vitamin E was not indicated [17]. In a study examining γ -tocopherol modulation of 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 lung eosinophils at day 4 after OVA challenge [122]. However, since 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 at 4 days after the last OVA challenge was

during the resolution phase of eosinophil inflammation. In mice deficient in liver α -tocopherol transfer protein (α TTP), they exhibit severe deficiency in tissue α - and γ -tocopherol as well as reduced IgE and reduced IL-5 after OVA challenge to the lung [123]. In these mice, it is not known whether severe tocopherol deficiency during mouse development alters leukocyte hematopoiesis or leukocyte responsiveness.

Tocopherols also regulate lung inflammation induced by LPS. Aerosol α -tocopherol (30ug/rat) administered immediately before inhalation of LPS reduces lung neutrophilia and reduces TNF α and cytokine-induced neutrophil chemoattractant-1 (CINC-1) [124]. However, the source and purity of the α -tocopherol as well as lung tissue levels of tocopherols were not determined [124]. It is also reported that intraperitoneal administered liposomes containing α -tocopherol (50 mg α -tocopherol/kg mouse) reduces LPS-induced lung neutrophilia without altering blood neutrophil numbers, TNF α , IL-1 β or MIP-1 α [125]. Furthermore, nebulized γ -tocopherol reduces neutrophilia, IL-8 and IL-6 in burn and smoke inhalation injury in sheep [126]. In summary, differences among the reports of tocopherol regulation of lung inflammation likely reflect differences in the isoforms of tocopherols, tocopherol isoform concentrations and time of administration of tocopherols.

Reports conflict regarding tocopherol regulation of mediators of inflammation

A. Tocopherol regulation of signal transduction

Reports conflict as to whether tocopherols modulate mediators of inflammation including prostaglandins, cytokines, chemokines and adhesion molecules [22, 98, 120, 127-142]. As antioxidants, the antioxidant capacity/mole of α -tocopherol and γ -tocopherol are relatively similar with regards to scavenging reactive oxygen species in vitro [98]. However, γ -tocopherol levels in tissues are 10% of that α -tocopherol. Moreover, γ -tocopherol, in contrast to α -tocopherol, reacts with reactive nitrogen species such as peroxynitrite forming 5-nitro- γ -tocopherol [8, 9]. Furthermore, when tocopherols are oxidized, they are recycled by reduction by vitamin C [12-14]. Tocopherols also have non-antioxidant functions by binding directly to enzymes in signal transduction pathways and regulating their enzymatic activity [143-145]. Thus, tocopherols have both antioxidant and non-antioxidant activities that could modulate cell signal transduction. Several non-antioxidant functions for tocopherols have been previously reviewed [146]. The non-antioxidant functions are primarily through regulation of enzymes that mediate signal transduction. Tocopherols inhibit several enzymes including protein kinase C (PKC), protein kinase B (PKB), protein tyrosine kinase, lipoxygenases (5-LOX, 12-LOX and 15-LOX), mitogen activated protein kinases (MAPKs), phospholipase A2 (PLA2), and cyclooxygenase-2 (COX-2) [146, 147]. In contrast, tocopherols activate several enzymes including protein phosphatase 2A (PP2A), protein tyrosine phosphatase, and diacylglycerol kinase [146].

There are reports suggesting tocopherol isoform specific regulation of cyclooxygenase generation of prostaglandin E2 (PGE₂). For example, γ -tocopherol but not α -tocopherol is able to inhibit COX-2-mediated PGE₂ synthesis in macrophages, Caco2 cells, and epithelial cells in vitro [21, 131, 132, 148-150] and γ -tocopherol inhibits eicosanoid synthesis in carrageenan-induced inflammation in rats [132]. Other investigators also report that α -tocopherol does not inhibit PGE₂ synthesis in macrophages and epithelial cells [129, 130, 134]. In contrast, some reports indicate that α -tocopherol inhibits PGE₂ synthesis in vitro in macrophages, endothelial cells, and microglia [127, 128, 133, 141]. The tocopherol metabolites CEHCs are also reported to modulate PGE₂ production. α -CEHC and γ -CEHC inhibit LPS-stimulated microglial PGE₂ and nitrite production [22]. Furthermore, α -CEHC decreases TNF α -stimulated nitrite production in endothelial cells and microglial cells [22].

In *in vivo* studies, we reported that in OVA-induced allergic inflammation *in vivo*, supplemental d- α -tocopherol and d- γ -tocopherol do not inhibit PGE₂ synthesis when physiological levels of purified tocopherols are administered after sensitization and before challenge with OVA [26].

Tocopherols are also reported to regulate lipoxygenase, PKC, NF κ B and the MAPK pathways. In monocytes, α -tocopherol directly binds to 5-LOX and 15-LOX and inhibits their activity [143, 144]. It is also reported that α -tocotrienol binds to 12-LOX and inhibits its activity [145]. With regards to PKC signaling, α -tocopherol blocks activation of protein kinase C and blocks hydrogen peroxide activation of NF κ B in several cell types [26, 147]. However, in another report, synthetic α -tocopherol-succinate but not α -tocopherol blocked TNF α activation of NF κ B in monocytes [151]. In lung epithelial cells, α -tocopherol blocks TNF α activation of NF κ B, MAPK, and ERK [152]. During cell growth, α -tocopherol inhibits PKC α by activating a phosphatase [25]. It is also reported that α -tocopherol decreases PKC phosphorylation of the p47phox subunit of NADPH oxidase in human monocytes and thus reduces translocation of p47phox to the membrane as well as the NADPH oxidase generation of ROS [153]. Since these signaling pathways, that have been examined *in vitro*, are involved in responses by leukocytes and endothelial cells, they may also have a role *in vivo* in non-antioxidant regulation of inflammation.

Tocotrienols also regulate signal transduction, although tocotrienols have been mainly studied for their anti-cancer and neuroprotective functions [154]. It is reported that tocotrienols but not tocopherols reduce VEGF-induced vessel formation by downregulating endothelial cell expression of the VEGF receptor, thereby reducing tumor cell growth [154, 155]. In neurons, tocotrienols down regulate Src, MAPKs and ERKs in response to H₂O₂ or glutamate treatment [154]. Moreover, α -tocotrienol and α -tocopherol bind directly to 5-lipoxygenase (5-Lox) and inhibit 5-Lox independent of its antioxidant property [154]. It is also reported that tocotrienols inhibit monocyte binding to endothelial cells and inhibit induction of expression of endothelial cell adhesion molecules including VCAM-1 [155-159]. Furthermore, α -tocopherol blocks TNF α activation of MAPK/ERK pathway in airway epithelial cells [152]. Since leukocyte recruitment, angiogenesis, 5-Lox, and signaling through Src/MAPK/ERK pathways are all important functions in inflammatory responses, tocotrienols may influence inflammation *in vivo*. However, it remains to be determined whether physiological levels of tocotrienols regulate inflammation *in vivo*. In summary tocopherols and tocotrienols can regulate signal transduction.

B. Tocopherol regulation of cytokine expression

With regards to α -tocopherol modulation of cytokines during inflammation, reports vary as to whether α -tocopherol affects cytokine expression in animal models of asthma and atherosclerosis [98, 120, 130, 140, 142]. In *in vitro* studies, α -tocopherol has also been reported to modulate cytokine production and lymphocyte proliferation. It is reported that lymphocyte proliferation in response to mitogen is increased by tocopherols with the following order of potency: β -tocopherol, δ -tocopherol, and then α -tocopherol [141]. Furthermore, γ -tocopherol and δ -tocopherol increase production of the cytokine IL-2 whereas α -tocopherol and β -tocopherol do not affect IL-2 production in response to mitogen *in vitro* [141]. With regards to chemokine production, Meydani's group has reported that α -tocopherol does not inhibit spontaneous production of the chemokine MCP-1 by endothelial cells *in vitro* [136]. We report that purified natural d- α -tocopherol and d- γ -tocopherol do not alter endothelial cell production of the chemokine MCP-1 [26]. We also reported that in experimental asthma, purified d- α -tocopherol and d- γ -tocopherol do not alter expression of the cytokines IL-4, IL-5, IFN γ , and IL-2 in bronchoalveolar lavage and do not alter expression of the chemokine eotaxin in lung digests [26]. In human volunteers, α -tocopherol supplementation does not alter MCP-1-activated monocyte production of IL-1b, IL-6 or

TNF α [142]. Furthermore, in patients with Type 2 Diabetes that were administered α -tocopherol, mixed tocopherols or tocopherol-stripped soybean oil placebo, there was no effect of the tocopherols on IL-6, TNF α or MCP-1 [160]. However, unfortunately, in this clinical study, α -tocopherol or mixed tocopherols at 500mg/day increased blood pressure and heart rate in individuals with type 2 diabetes [104]. In summary, in vivo, α -tocopherol and γ -tocopherol can modulate inflammation without altering cytokine or chemokine expression. Moreover, in several reports on endothelial cell function in inflammation, leukocyte infiltration into tissue is altered without changes in expression of cytokines or chemokines [49, 91, 92]. Consistent with these reports on endothelial function in inflammation, we have reported that α -tocopherol and γ -tocopherol modulate endothelial cell signal transduction during leukocyte migration in vitro and modulate leukocyte recruitment in vivo without altering tissue expression of cytokines, chemokines or adhesion molecules [26].

C. Tocopherol regulation of adhesion molecule expression

Whether α -tocopherol modulates endothelial cell adhesion molecule expression in vitro varies in the literature. In vitro, α -tocopherol is reported to block IL-1 β -induced ICAM-1 expression on human aortic endothelial cells but not on human umbilical vein endothelial cells and then, in another report, α -tocopherol does not inhibit TNF- α -stimulated ICAM-1 expression on human umbilical vein endothelial cells [135, 136]. It is also reported that tocopherols block TNF α induction of VCAM-1 expression on immortalized dermal capillary endothelial cells in vitro [155]. However, in another report, TNF α -induced expression on endothelial cells was blocked by synthetic α -tocopherol-succinate but not by natural α -tocopherol [137]. We reported that physiological levels of natural α -tocopherol and γ -tocopherol do not alter VCAM-1 expression on endothelial cell lines in vitro [26]. For in vivo administration of supplemental physiological levels of tocopherols, we reported that purified natural d- α -tocopherol and natural d- γ -tocopherol do not alter OVA-induced VCAM-1 expression on lung venules [26]. Moreover, the mechanism for purified natural d- α -tocopherol and d- γ -tocopherol modulation of leukocyte migration is, at least in part, by a direct effect of these tocopherols on the endothelial cells. This is significant since endothelial cells actively function in the promotion of leukocyte transendothelial migration [50, 90, 161]. With regards to tocotrienol regulation of adhesion molecules in vitro, it is reported that tocotrienols have a 10 fold efficacy for inhibition of TNF α -induced VCAM-1 expression on human umbilical vein endothelial cells in vitro [156-158]. However, it is not known whether in vivo, physiological levels of tocotrienols regulate VCAM-1 expression.

We suggest that variations in reports on outcomes of vitamin E treatments in vitro and in vivo result, at least in part, from differences in isoforms and purity of tocopherols, in concentrations of the tocopherols within different cells, and in experimental systems. This is important considering our report indicating that forms of tocopherols have cell type-specific opposing regulatory functions on leukocyte recruitment since tocopherols directly affected endothelial cells but not leukocytes during leukocyte transmigration [26]. Moreover, our report that supplemental versus highly-elevated doses of natural γ -tocopherol have opposing functions (McCary et al, manuscript submitted) suggests that isoform doses used in studies in vitro and in vivo to examine physiological functions of tocopherols is critical for interpretations of outcomes.

Clinical implications for vitamin E regulation of lung inflammation

It is reported that patients with mild asthma have reduced α -tocopherol, reduced ascorbic acid, and increased glutathione in airway fluid but these patients have normal blood levels of tocopherol and ascorbic acid (vitamin C) [162]. In severe asthma, it is reported that there is reduced levels of the antioxidant bilirubin [117]. In other studies, asthmatic patients had

reduced sera α -tocopherol and ascorbic acid even during the asymptomatic periods of asthma [163]. In animal studies, the α -tocopherol and ascorbic acid are decreased in broncoalveolar lavage of guinea pigs sensitized with OVA [164]. Therefore, it has been suggested that supplementation with vitamins E and C may regulate lung inflammation.

Reports of clinical studies on vitamin E primarily focus on the α -tocopherol isoform without adjustment for the dietary contribution of γ -tocopherol to the outcomes of these studies. For interpretation of the clinical studies, it is especially important to take into consideration the dietary contribution of tocopherol isoforms because γ -tocopherol is more abundant in western diets. The average plasma concentration of α -tocopherol is the same among many countries [24]. However, the American diet is rich in γ -tocopherol found in soy oil, the major form of vegetable oil in the United States. In contrast, γ -tocopherol is low in other oils (sunflower and olive oil) commonly used in some of the European countries (Figure 7) [21, 24, 26]. However, as countries assume western lifestyles, diets change including increased consumption of soybean oil [165]. Consistent with this, in the United States and the Netherlands, the average plasma γ -tocopherol level is 2-6 times higher than that reported for 6 European countries including Italy (Table 1) [24]. This fold increase in plasma γ -tocopherol is similar to fold increase in plasma γ -tocopherol in the animal studies [26] in which γ -tocopherol elevated allergic inflammation and γ -tocopherol opposed the anti-inflammatory functions of α -tocopherol, even at 1/10 the concentration of α -tocopherol.

A consistent feature of inflammation in allergic asthma is the recruitment of eosinophils and mast cells. The recruitment of these cells is regulated by VCAM-1 and tocopherols [26, 53-60]. In clinical studies of asthma, it is reported 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 United States or the Netherlands [166-170]. These clinical outcomes are consistent with an interpretation that there is little benefit of α -tocopherol for inflammation in the presence of 2-6 fold elevation in plasma γ -tocopherol in people in the United States and the Netherlands (Table 1). Therefore, differences in outcome of the clinical reports on vitamin E modulation of asthma in European countries and the United States may, in part, reflect the opposing regulatory functions of α - and γ -tocopherol forms of vitamin E consumed in diets and supplements. In Israel, it is reported that vitamin E supplementation reduces nasal symptoms of seasonal ragweed allergic rhinitis, although the form and purity of vitamin E and the contents of the placebo were not indicated [171]. 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 [169, 172-176], the clinical data are consistent with the animal studies demonstrating opposing functions of the tocopherol isoforms on leukocyte recruitment [26].

It has also been suggested that changes in environmental factors including vitamin E consumption may contribute to the increased incidence of asthma. The incidence of asthma in several countries including the United States and the Netherlands has dramatically increased in the last 40 years [177-179]. It is thought that there must be environmental factors contributing to this increase since it is too rapid for genetic changes. The prevalence of asthma is higher in the United States than Western Europe or Mediterranean countries [180]. 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 [181]. The increases in prevalence occur as countries assume western lifestyles [181]. The dietary changes in the United States in the last 40 years with increased consumption of γ -tocopherol in vegetable oil may, in part, be a contributing factor to changes in asthma prevalence. In addition, in a Scottish cohort, it is reported that reduced maternal intake of vitamin E (likely referring to α -tocopherol) is

associated with increased asthma and wheezing in children up to 5 years old [182]. Then in this same report, it was discussed that from 1967 to 2004, there was a significant increase in vegetable oil intake by Scottish [182], which we interpret as indicative of an increase in dietary γ -tocopherol since vegetable oil (soy oil) is rich in γ -tocopherol (Figure 7). In a study in the United Kingdom, α -tocopherol administration in soybean oil to asthmatics did not have benefit for asthmatics [183]. This is consistent with the interpretation that the γ -tocopherol in soybean oil ablates the benefit of α -tocopherol supplementation. In addition, the isoforms of vitamin E in the patients are not indicated in these studies [183]. In summary, since α -tocopherol levels and other antioxidants are low in asthmatics [117, 162-164, 184, 185] and since α -tocopherol can reduce inflammation, an increase in physiological levels of 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. Furthermore, considering the studies in Figure 6 on the reversibility of tocopherol effects, clearance of γ -tocopherol and supplementation with α -tocopherol are complex and, in animal studies, only complete inhibition of inflammation was obtained at very high doses of tocopherols. In addition, there is caution for high doses of tocopherols affecting vascular function and increasing the incidence of high blood pressure, hemorrhagic stroke, all-cause mortality, or post trial cerebral infarction [104-109].

Clinical implications for vitamin E regulation of inflammation in osteoarthritis and atherosclerosis

The tocopherol isoform levels may also affect inflammation in other diseases that involve VCAM-1 such as osteoarthritis and atherosclerosis [70, 73, 186-188]. Although there are conflicting reports for tocopherol regulation in these diseases, we suggest that this may result from opposing functions of tocopherol isoforms present in the subjects in these studies. It has been reported that plasma γ -tocopherol is positively associated with osteoarthritis whereas plasma α -tocopherol is inversely associated with osteoarthritis [189]. In contrast, in another report on knee osteoarthritis in patients from Australia, vitamin E supplementation (α -tocopherol) did not relieve symptoms but they did not measure α -tocopherol or γ -tocopherol levels [190]. With regards to coronary heart disease and stroke, the benefit of tocopherols are also inconsistent among the studies [18, 191-193]; furthermore, measurement of levels of both α -tocopherol and γ -tocopherol in tissues are commonly not reported [98, 191, 192, 194-196]. Studies of tocopherols and heart disease are complex since different dietary oils not only contain different forms of tocopherols but also contain different lipids that affect heart disease. Nevertheless, 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 [191]). In contrast, α -tocopherol intake is either not associated with heart disease or, in other reports, is associated with reduced death from heart disease [192, 194-196]. Devaraj et al [197] report that high dose α -tocopherol supplementation (800mg/day for 2 years) raised human plasma α -tocopherol from 23 μ M to 36 μ M but did not alter carotid intimal medial thickness in patients with coronary artery disease. However, γ -tocopherol was not measured in these patients and, importantly, the placebo control for this study was soybean oil which is rich in γ -tocopherol (Figure 7). Therefore, these studies could be interpreted as consistent with the presence of pro-inflammatory levels of γ -tocopherol opposing an inhibitory effect of α -tocopherol on atherosclerosis. In a study of insulin-resistant rats on high fructose diets, γ -tocopherol but not α -tocopherol inhibited accumulation of 3-nitrotyrosine in neointima and reduced neointimal formation induced by vascular injury, but superoxide production was not altered by the tocopherols [198]. However, in this study the tocopherols were delivered in corn oil which contains mixed tocopherols (Figure 7) and they report that when each tocopherol was administered there were increases in both α -tocopherol and γ -

tocopherol in the rat plasma [198]. Furthermore, in these studies, plasma γ -tocopherol was highly elevated by 10 fold whereas plasma α -tocopherol was elevated only 2 fold [198]. Thus, our interpretation is that this study may indicate that with very high γ -tocopherol in the presence of modest levels of α -tocopherol, there is inhibition of carotid neointimal formation [198]. This is consistent with our recent studies that highly elevated levels of γ -tocopherol are anti-inflammatory whereas supplemental levels of γ -tocopherol are pro-inflammatory [26] (McCary et al, manuscript submitted). Consistent with tocopherol regulation of atherosclerosis, in α -tocopherol transfer protein deficient mice, which exhibit a deficiency in both α -tocopherol and γ -tocopherol in plasma [199], there is increased lipid peroxidation and increased severity of atherosclerosis [200]. In summary, we suggest that although the clinical reports on vitamin E association with heart disease are inconsistent, for those reports with an effect on heart disease, supplemental γ -tocopherol is associated with an increase in heart disease whereas α -tocopherol or perhaps highly-elevated γ -tocopherol is associated with a decrease in parameters of heart disease. Therefore, the opposing functions of α -tocopherol and γ -tocopherols in animal models [26] are consistent with the different outcomes for the clinical studies of tocopherols in heart disease. With regards to the tocotrienols, it is not known whether the purified tocotrienol isoforms regulate inflammatory disease in vivo. However, it is reported that mixed tocotrienols in palm oil, that contains different lipids than the control groups, limit atherosclerosis in animal models [201]. It is also reported that, in contrast to tocopherols, tocotrienols are neuroprotective and have anti-cancer properties [154, 202], suggesting again that the vitamin E forms have differential regulatory properties. Future clinical studies of vitamin E regulation of inflammatory diseases should include a systematic design to examine opposing functions of the isoforms and levels of vitamin E on inflammation, leukocyte recruitment and disease parameters.

Concluding Remarks

Vitamin E regulation of disease has been extensively studied in humans, animal models and cell systems. Most of these studies focus on the α -tocopherol isoform of vitamin E. These reports indicate contradictory outcomes for anti-inflammatory functions of the α -tocopherol isoform of vitamin E, especially with regards to clinical studies of asthma and atherosclerosis. These seemingly disparate clinical results are consistent with recently reported unrecognized properties of isoforms of vitamin E. Specifically, it has recently been reported that supplementation with physiological levels of purified natural forms of the vitamin E isoforms α -tocopherol and γ -tocopherol has opposing regulatory functions during inflammation such that α -tocopherol is anti-inflammatory and γ -tocopherol is pro-inflammatory. Interestingly, treating with supplemental tocopherols and then switching the tocopherol form to supplemental levels of a functionally opposing tocopherol does not completely reverse the OVA-induced inflammatory phenotype. Moreover, this partial reversal of inflammation by switching the administration of isoforms of tocopherol has important implications for clinical studies with tocopherol supplements. In contrast, highly elevated α -tocopherol can reverse the pro-inflammatory effects of supplemental levels of γ -tocopherol. However, although raising the tissue plasma α -tocopherol and γ -tocopherol levels 50 fold (highly-elevated tocopherols) is anti-inflammatory and can reverse the pro-inflammatory effects of supplemental levels of γ -tocopherol, clinical reports caution that elevation of tocopherols can increase the incidence of high blood pressure, hemorrhagic stroke, all-cause mortality, or post trial cerebral infarction [104-109]. In summary, the differential regulation of inflammation by isoforms of vitamin E provide a basis towards designing drugs and diets that more effectively modulate inflammatory pathways and improve health.

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LIST OF ABBREVIATIONS

BAL	bronchoalveolar lavage
CEHC	carboxyethyl-hydroxychroman
COX2	cyclooxygenase-2
Gαi	G protein α i
ICAM-1	intercellular adhesion molecule-1
IFNγ	interferon-gamma
IL	interleukin
LCMV	lymphocytic choriomeningitis virus
LPS	lipopolysaccharide
Lox	lipxygenase
MadCAM-1	mucosal addressin cell adhesion molecule-1
MAPK	mitogen activated protein kinases
MMP	matrix metalloproteinase
NFκB	nuclear factor kappa B
OVA	chicken egg ovalbumin
PECAM-1	platelet-endothelial cell adhesion molecule-1
PKCα	protein kinase C α
PLA2	phospholipase A2
PP2A	protein phosphatase 2A
PTP1B	protein tyrosine phosphatase 1B
ROS	reactive oxygen species
VCAM-1	vascular cell adhesion molecule-1
TIMP	tissue inhibitor of metalloproteinase
TNFα	tumor necrosis factor-alpha
αTTP	alpha-tocopherol transfer protein
VCAM-1	vascular cell adhesion molecule-1

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Figure 1. Natural R,R,R-tocopherols and R,R,R-tocotrienols

The isoforms differ in the number of methyl groups on the chromanol head group. The α-tocopherol isoform is the most abundant in tissues. The γ-tocopherol isoform is abundant in the diet but, in tissues, γ-tocopherol is 10 fold less abundant than α-tocopherol because of preferential transfer of α-tocopherol in the liver by α-TTP. The other forms of tocopherols and tocotrienols are less abundant in the diet and in tissues than α-tocopherol and γ-tocopherol.

Figure 2. Leukocyte transendothelial migration

During inflammation, cytokines produced in the tissue induce endothelial cell adhesion molecule expression. In addition, chemoattractants released by both the tissue and endothelial cells increase leukocyte adhesion molecule affinity as well as provide direction for leukocyte migration. Leukocyte binding to the endothelial cell adhesion molecules activates intracellular signaling in the endothelial cells for the formation of small passageways through which leukocytes migrate. L, leukocyte.

Figure 3. VCAM-1 signal transduction

VCAM-1 is located on the luminal and lateral but not the basal surface of vascular endothelial cells in tissues. Crosslinking of VCAM-1 activates calcium fluxes and Rac-1 which then activates endothelial cell NOX2. NOX2 catalyzes the production of superoxide that then dismutates to H_2O_2 . VCAM-1 induces the production of only $1 \mu M$ H_2O_2 . Within minutes of its production, H_2O_2 activates endothelial cell-associated matrix metalloproteinases (MMPs) that degrade extracellular matrix and endothelial cell surface receptors in cell junctions. The endothelial cell-derived H_2O_2 also mediates a 2-5 hr delayed activation of lymphocyte-associated MMPs by inducing the degradation of leukocyte tissue inhibitors of metalloproteinases (TIMPs). H_2O_2 also diffuses through membranes at $100 \mu m/sec$ to oxidize and transiently activate endothelial cell protein kinase C- α (PKC α). PKC α phosphorylates and activates protein tyrosine phosphatase 1B (PTP1B). PTP1B is not oxidized. These signals through reactive oxygen species (ROS), MMPs, PKC α , and PTP1B are required for VCAM-1-dependent leukocyte transendothelial migration. The G protein G αi in endothelial cells is also involved in VCAM-1 signaling and leukocyte transendothelial migration.

Figure 4. Tocopherol regulation of VCAM-1-induced reactive oxygen species

Tocopherols regulate ROS generation and PKC α during VCAM-1 signaling [26].

Tocopherols are lipids in the plasma membrane. The tocopherol chromanol head group is external to the membrane and is thus poised for scavenging of extracellular reactive oxygen species. Furthermore, tocopherols are found in membranes of organelles and can scavenge intracellular reactive oxygen species. Tocopherols in membranes also regulate activation of PKC α that occurs at membranes. There is approximately 10 fold more α -tocopherol than γ -tocopherol in membranes in vivo. Interestingly, at physiological levels of tocopherols, α -tocopherol inhibits VCAM-1 signaling whereas γ -tocopherol ablates the inhibition by α -tocopherol [26].

Figure 5. VCAM-1-dependent eosinophil recruitment during allergic lung inflammation

After intraperitoneal sensitization with the antigen OVA in the adjuvant alum, the lung is challenged with OVA. In this model, eosinophil recruitment from the blood is blocked with anti-VCAM-1 blocking antibodies. In contrast, lymphocytes, monocytes and neutrophils migrate on ICAM-1 or PECAM-1. After the leukocytes undergo transendothelial migration, the leukocytes migrate through the tissue, across the epithelium and into the airway spaces. Chemokines in the tissue direct the leukocyte migration.

References: (26) and McCary et al (manuscript submitted)

Figure 6. Tocopherol regulation of lung lavage inflammation

Following antigen sensitization and subsequent supplemental subcutaneous tocopherol treatments and OVA challenges in phase 1 [26], mice entered a 4-week clearance phase during which they were not treated with tocopherols or challenged with OVA. To examine reversibility of the effects of α -tocopherol and γ -tocopherol, in a second phase (phase 2), mice were again treated with daily subcutaneous doses of tocopherol as indicated and re-challenged with OVA. Note that additional treatment groups received highly-elevated tocopherol (10x the supplemental level of tocopherols). Mice were sacrificed and inflammation in the lung lavage was examined either at the end of phase 1 (day 21) or the end of phase 2 (day 54). α T, α -tocopherol; γ T, γ -tocopherol; i.n., intranasal; i.p., intraperitoneal.

Figure 7. α -tocopherol and β -tocopherol in dietary oils

Adapted from [26]. Tocopherols were extracted from dietary oils and measured by HPLC with an electrochemical detector.

Table 1

Human plasma tocopherol

Human plasma:	γ T (μ M)	α T (μ M)
USA (4 reports)	2.5	22
	5.4	22
	5.2	27
	7	20
Netherlands	2.3	25
France	1.2	26
Ireland	1.8	26
Italy	1.2	24
Austria	1.4	21
Ireland	1.8	26
Spain	1.7	27
Lithuania	1.6	22

Adapted from Wagner et al., 2004 *Annals Nutr Metab* 48:169