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Journal of Nutritional Biochemistry 21 (2010) 589–597
Journal of Nutritional Biochemistry

α-, γ- and δ-tocopherols reduce inflammatory angiogenesis in human microvascular endothelial cells☆

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Received 16 February 2009; received in revised form 10 March 2009; accepted 17 March 2009

Abstract

Vitamin E, a micronutrient (comprising α-, β-, γ- and δ-tocopherols, α-, β-, γ- and δ-tocotrienols), has documented antioxidant and non-antioxidant effects, some of which inhibit inflammation and angiogenesis. We compared the abilities of α-, γ- and δ-tocopherols to regulate human blood cytotoxicity (BEC) and lymphatic endothelial cytotoxicity (LEC), proliferation, invasiveness, permeability, capillary formation and suppression of TNF-α-induced VCAM-1 as in vitro models of inflammatory angiogenesis. α-, γ- and δ-tocopherols were not toxic to either cell type up to 40 μM. In BEC, confluent cell density was decreased by all concentrations of δ- and γ-tocopherol (10–40 μM) but not by α-tocopherol. LEC showed no change in cell density in response to tocopherols. δ-Tocopherol (40 μM), but not other isomers, decreased BEC invasiveness. In LEC, all doses of γ-tocopherol, as well as the highest dose of α-tocopherol (40 μM), decreased cell invasiveness. δ-Tocopherol had no effect on LEC invasiveness at any molarity. δ-Tocopherol dose dependently increased cell permeability at 48 h in BEC and LEC; α- and γ-tocopherols showed slight effects. Capillary tube formation was decreased by high dose (40 μM) concentrations of α-, γ- and δ-tocopherol, but showed no effects with smaller doses (10–20 μM) in BEC. γ-Tocopherol (10–20 μM) and α-tocopherol (10 μM), but not δ-tocopherol, increased LEC capillary tube formation. Lastly, in BEC, α-, γ- and δ-tocopherol each dose-dependently reduced TNF-α-induced expression of VCAM-1. In LEC, there was no significant change to TNF-α-induced VCAM-1 expression with any concentration of α-, γ- or δ-tocopherol. These data demonstrate that physiological levels (0–40 μM) of α-, γ- and δ-tocopherols are nontoxic and dietary tocopherols, especially δ-tocopherol, can limit several BEC and LEC endothelial behaviors associated with angiogenesis. Tocopherols may therefore represent important nutrient-signals that limit cell behaviors related to inflammation/angiogenesis, which when deficient, may predispose individuals to risks associated with elevated angiogenesis such as inflammation and cancer; further differences seen from the tocopherols may be due to their blood or lymphatic cell origin.
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Keywords: Tocopherol; Vitamin-E; Angiogenesis; Inflammation; Lymphatic; Endothelial; VCAM-1

1. Introduction

Vitamin E is an essential fat-soluble antioxidant nutrient that balances pro- and antioxidant reactions in tissues [1]. Vitamin E exists in nature as eight vitamers, that is, four tocopherols (RRR-α, -β, -γ and -δ tocopherols) and four tocotrienols (RRR-α, -β, -γ and -δ tocotrienols). All isomers exhibit strong antioxidant activities [2]. Most tocopherol studies have focused on α-tocopherol; fewer studies have examined γ- and δ-tocopherols. Saeed et al. [3] showed that α-tocopherol reduced formation of lipid peroxides (TBARS) compared to controls. Recent studies indicate that γ-tocopherol, but not α-tocopherol, attenuate the destructive effects of $NO^{\cdot}/ONOO^{-}$ toward oxidant sensitive enzymes, for example, α-ketoglutarate dehydrogenase [4]. γ-Tocopherol nitration may be an important mechanism for removal of toxic reactive nitrogen species in vivo. Tocopherols and tocotrienols may also modify the activities of oxidant-regulated transcription factors (e.g., NF-κB) to modulate levels of many genes involved in inflammation and also angiogenesis [5–7]. The antioxidant properties of tocopherols reduce the expression of integrins, ICAM-1 and VCAM-1, which may reduce inflammation and angiogenesis [7–10]. There is also emerging evidence that vitamin E could also function through oxidant-independent mechanisms such as inhibiting PKC and activating protein phosphatase 2A [11].

Angiogenesis, the growth of new blood vessels, has long been recognized as an essential step in the development of tumor progression [12] and, most recently, in inflammation [13]. Inflammatory angiogenesis is a characteristic of several acute and chronic conditions such as inflammatory bowel disease, arthritis and psoriasis [14]. Oxidant-regulated cell signaling mechanisms are active during

☆ These studies were supported by funding through Yasoo Health Inc., a grant from the United Nations (UNESCO) and NIH DK43785.
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0955-2863/$ — see front matter. Published by Elsevier Inc.
doi:10.1016/j.jnutbio.2009.03.006

inflammatory and angiogenic conditions which are both characterized by the proliferation and activation of microvascular endothelial cells, and increased endothelial adhesion molecule expression (VCAM-1, ICAM-1). For example, α-tocopherol inhibits VCAM- and ICAM-dependent leukocyte adhesion inflammation [15–17]. Vitamin E inhibits angiogenesis in bovine aortic endothelial cells [18], as well as in human endothelial cells via IL-8 [19], VEGF [20] and NF-κB signal suppression [6]. In this report, we investigated anti-angiogenic and anti-inflammatory properties of α-, γ- and δ-tocopherols on human blood vessel and lymphatic endothelial cells. Our data indicate that tocopherols (δ-tocopherol in particular) suppress many inflammatory and angiogenic activities making them potentially useful against inflammatory bowel disease, cancer, arthritis, psoriasis and cardiovascular disease.

2. Methods

2.1. Endothelial cell culture and tocopherol treatment

Immortalized human dermal capillary cells, HMEC-1, were provided as a generous gift from Dr. Gerald Ades and Francisco Candal, CDC (Emory University, Atlanta, GA). HMEC-1A is a subcloned population of pure lymphatic endothelial cells, which was used as a model of lymphatic endothelial cell responses. HMEC-1 as well as HMEC-1A is cultured to confluency in MCBD131 medium supplemented with 10% heat-inactivated FBS (HI FBS), 1% penicillin/streptomycin/amphotericin (PSA), 10 ng/ml EGF and 1 μg/ml hydrocortisone (HC). Tocopherols as ethanol stock solutions (D-α-tocopherol, D-δ-tocopherol and D-γ-tocopherol) were added to medium to 10, 20, 40 and 100 μM; vehicle concentrations never exceeded 0.1% ethanol.

2.2. Cytotoxicity assay

To test HMEC for cell viability and toxicity in response to tocopherols, 96-well plates were coated with 2% gelatin prior to set up. HMECs were cultured to confluency in MCBD131 medium without HC. The medium was aspirated, and 100 μl of the control and 100 μl of diluted concentrations was added per well and incubated at 37°C for 48 h. One hundred percent cell detachment was accomplished by treating wells with 0.1% NH_4OH in water to lift cells and leave matrix. NH_4OH was added, mixed thoroughly to loosen the cells and aspirated. All wells but eight were then stained with crystal violet, washed with 1× PBS and then left to air-dry. Crystal violet (0.01% crystal violet, in 3.7% phosphate-buffered formaldehyde) was added, left at room temperature for 30 min, washed once with 1× PBS and left to air-dry. The absorbance was read by a Multiskan MCC/340 Titertek at 540 nm.

2.3. Cell proliferation

Cell growth and inhibition assays were set up by seeding HMEC at 5% confluent density in 2% gelatin-coated 96-well plates, in complete medium (100 μl/well). Medium containing α-, γ- or δ-tocopherol was added to wells after cells had attached and the plates were incubated at 37°C for 72 h. The medium was removed by aspiration, and 0.1% crystal violet in 3.7% phosphate-buffered formaldehyde was added to each well and allowed to fix at room temperature for 30 min. Plates were washed 1× with PBS, aspirated and air-dried; the absorbance was read at 540 nm.

2.4. Capillary tube formation

HMEC-1 and HMEC-1A were cultured to confluency in MCBD131 medium without HC and placed in 37°C incubator. Aliquots of Matrigel were placed on ice at 4°C overnight. One hundred-microliter aliquots of Matrigel were transferred into each precooled 96-well plate; the plate was then sealed and incubated at −20°C. The plate was transferred from a −20°C to a 4°C environment 24 h before tocopherol addition. Ninety microliters of cells and 10 μl of the diluted tocopherol were added to the 100 μl Matrigel. The plate was incubated at 37°C and pictures were taken at 24, 48 and 72 h with a Nikon Coolpix 990 camera and were analyzed using ImageJ.

2.5. Endothelial invasiveness

In the wound healing studies, HMEC-1 and HMEC-1A cells were grown to confluency in complete medium in 96-well plates. Circular wounds were eroded in a monolayer of HMEC cells using a rotating silicone rubber cone at approximately 2500 rpm (Becton Dickson no. 309602). The rubber polishing tip (2 mm diameter) was fitted to a MultiPro Model 780 lathe (Dremel) on a spring-loaded support stand. After wounding, detached cells were aspirated and fresh treatment medium containing tocopherols were added to the wells. Wound recovery was measured 24, 48 and 72 h later. The medium was removed by aspiration followed by the addition 0.1% crystal violet in phosphate-buffered formaldehyde (0.01% crystal violet, in 3.7% phosphate-buffered formaldehyde). The cells were incubated for 30 min, washed once with PBS and aspirated. Plates were air-dried, and absorbance was read at 540 nm. Wounding was measured as the fraction of recovered area compared to untreated controls (using absorbance of stained plates).

2.6. Cell ELISA assay for TNF-α-induced VCAM-1

For cell ELISA studies, the central 24 wells of 48-well plates were coated with 2% porcine skin gelatin and then seeded with HMEC-1 or HMEC-1A equivalent to 100% density. MCDB131 medium with 10% HI FBS, PSA and EGF, minus HC, was used. Cells were incubated for 24 h prior to stimulation. The growth medium was removed by aspiration, and treatment media (media with α-, γ- or δ-tocopherol at 10, 20 or 40 μM) was added to the monolayers (n=4). After 24 h preincubation in tocopherols, TNF-α at a concentration of 20 ng/ml was added to all wells except for four control wells, and the cells were incubated for an additional 16 h. Treatment media were removed and each well washed 3× with HBSS/PBS/1% FBS (H/P/1F). Antihuman VCAM-1 (Cell Sciences, MA) diluted 1:200 in HBSS/PBS/5% FBS (H/P/5F) was added to each well and incubated at 37°C for 30 min. Antibody was removed by aspiration, and each well washed twice with H/P/1F. Horseradish peroxidase-conjugated antimouse secondary antibody, diluted 1:2000 in H/P/5F, was added to each well and incubated at 37°C for an additional 30 min. The wells were then washed 4× with H/P/1F, and 250 μl HRP substrate solution (40 ml water, 4 μl 30% H_2O_2, 400 μl of 10 mg/ml tetramethylbenzidine in acetone) was added to each well. Following incubation at 37°C for ~30 min, the color reaction was stopped by the addition of 75 μl of 8N H_2SO_4. Two hundred microliters of each sample was transferred to a 96-well plate, and absorbance was read at 450 nm. TNF-α-induced expression was set as the "maximal" VCAM-1 induction compared to untreated controls. Fractional reductions in the induction of VCAM-1 were analyzed by one-way ANOVA with Bonferroni posttesting.

2.7. Trans-endothelial electrical resistance

For the trans-endothelial electrical resistance (TEER) studies, inserts with 8 μm pore size were placed into 24-well plates and coated with 2% gelatin. One milliliter of complete medium was added to each outer well and 100,000 cells were added in 500 μl of complete medium in each insert. Cells were allowed to grow to confluency (approximately 4 days) at 37°C. After cells were confluent, a baseline reading was taken and the medium was changed to treatment medium with tocopherols (1 h after readings were taken). α-, γ- or δ-tocopherol was added to both the insert and the well so that the concentrations in the inserts and the wells were the same. TEER readings were taken at 24, 48 and 72 h using an EVOM voltohmeter (WPI). The data were collected using Microsoft Excel and analyzed using one-way ANOVA with Dunnett's posttest using GraphPad InStat version 3.00.

3. Results

3.1. Cytotoxicity of tocopherols

High levels of tocopherols (50–200 μM) have a deleterious effect on specific cell types in vitro [15,16,21]. Depending on the solvent, serum concentration model system, and species, tocopherols can yield different effects. These differences are important to evaluate since in some assays, the absence of a biological effect can therefore be interpreted as "protection" but actually reflects cell stress or death; parallel analyses of toxicity are important controls. Previous studies have evaluated tocopherol toxicity through trypan blue staining [15,16]. This test only evaluates membrane integrity not changes in viability or survival. We examined cell responses in medium-supplemented with α-, γ- or δ-tocopherol under conditions where the tocopherol concentrations were below 50 μM, and the vehicle (solvent) concentration was at or below 0.1% ethanol. Our studies confirm that under these conditions, human microvessel endothelial cells are not affected either by tocopherols or by the solvent (using crystal violet uptake). However, above 50 μM, tocopherols were obviously and significantly toxic to cells between 4 and 24 h as observed by cell rounding and detachment.

In contrast to previous findings by Yoshida et al. [15,16], we found that HMEC-1 and HMEC-1A endothelial cells exhibited significant toxicity when exposed to levels at or greater than 50 μM α-, γ- or δ-tocopherol for time periods of over 4 h [15,16]. Evidence of toxicity occurred with tocopherols at 50–100 μM, at which point lytic, detergent-like properties of tocopherols are observed. Therefore, reductions in the apparent expression of

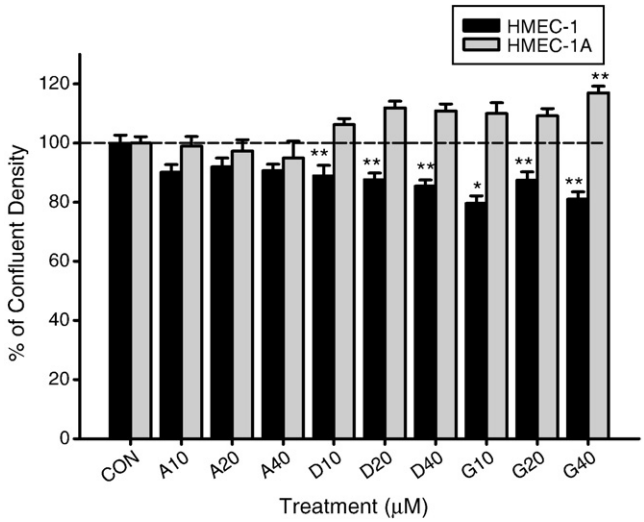

Fig. 1. Tocopherols decrease confluent density of blood but not lymphatic endothelial cells. HMEC-1 (blood endothelial cells) or HMEC-1A (lymphatic endothelial cells) were treated with different molarities of α-, γ- and δ-tocopherols at 24 h and compared to control. Results are expressed as a fraction of control confluent cell density for each cell type (black bars=HMEC-1, grey bars=HMEC-1A, error bars=S.E.M.). *P<.05, significantly different from control; **P<.01, significantly different from control using one-way ANOVA and Dunnett's multiple comparison.

endothelial adhesion molecules in response to inflammatory stimuli following exposure to oxidized LDL may incorrectly interpret diminished cell viability as protection. Cells exposed to high levels of tocopherols may not lyse but could leave them "stunned" or unresponsive to inflammatory stimuli due to membrane "stunning" based on membrane-altering properties of tocopherols at higher levels. With regard to species dependence, we found that murine endothelial cells [22] exhibit significant toxicity in response to levels of tocopherols that are well tolerated by human cells (~10 μM) (unpublished data).

3.2. Tocopherols reduce HMEC and HMEC-1A cell proliferation

α-, γ- and δ-tocopherols did not have a significant effect on cell viability up to 40 μM. Therefore, we evaluated tocopherol effects on cell proliferation using the MTT assay within the concentration ranges described. Cell proliferation was measured in complete medium containing α-, γ- or δ-tocopherol (0–40 μM). For HMEC-1, δ- and γ-tocopherol decreased cell proliferation consistent with an anti-angiogenic effect of these tocopherols. α-Tocopherol did not have a significant effect on cell density when compared to controls. γ-Tocopherol at 10 μM significantly reduced cell proliferation by 11.07% (Fig. 1) but to a lesser extent than δ-tocopherol. γ-Tocopherol at 20 and 40 μM reduced cell proliferation by 12.3% and 14.5%, respectively. Assays with δ-tocopherol demonstrated that the 10-, 20- and 40-μM treatments reduced confluent density by 20.3%, 12.5% and 18.9%, respectively (see Fig. 1). For lymphatic cells, γ-tocopherol at the highest concentration had a statistically significant effect, but α- and δ-tocopherols had no significant effect (Fig. 1).

3.3. Tocopherols modify capillary tube formation in blood vessels and lymphatic cells

Treatment of endothelial cells for 24 h with tocopherols at 40 μM reduced angiogenesis measured by capillary tube formation in HMEC-1 cells (Fig. 2) while the number of capillary tubes increased significantly for 1A cells (Fig. 3). Compared to controls, α-tocopherol at 20 and 40 μM reduced the number of capillary tubes by 13% (not shown) and by 25.4%, respectively, while it had no significant effect at 10 μM (not shown). γ- and δ-tocopherols (40 μM) produced similar results (24.7% and 22.4% reduction, respectively) but had no significant effect at 10 and 20 μM (not shown). In lymphatic cells, α-tocopherol at 10 μM and γ-tocopherol at 10, 20 and 40 μM increased the number of capillary tubes when compared to control (Con, 122.2±55.1; α10, 225.3±4.2; γ10, 278.3±37.9; γ20, 298.3±35.4; γ40, 241.5±82.7). δ-Tocopherol had no significant effects at any doses.

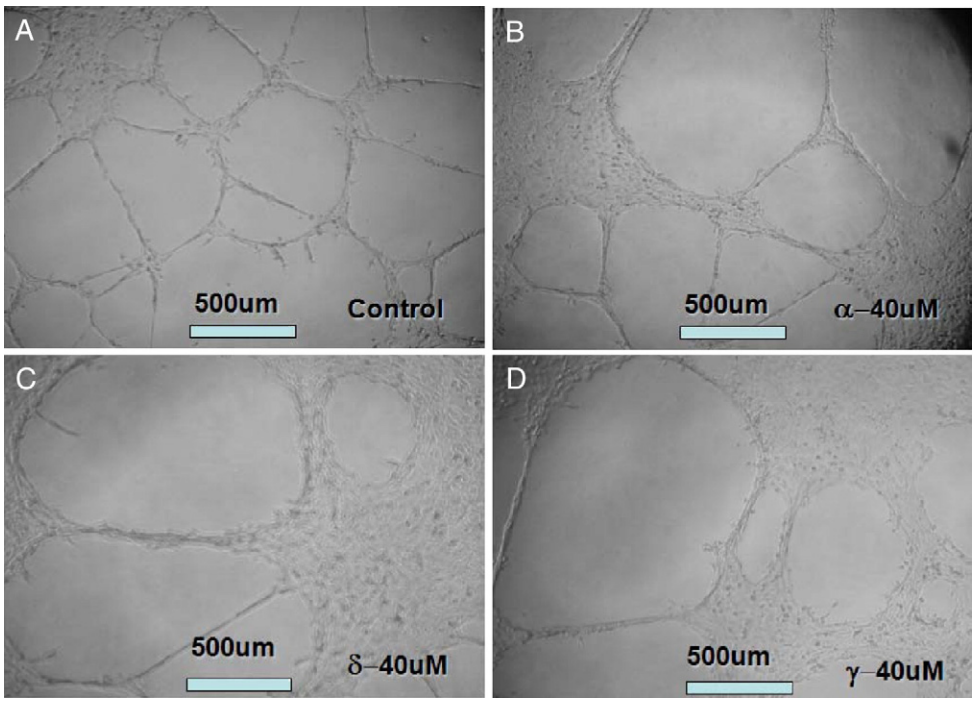

Fig. 2. Blood endothelial cell (HMEC-1) capillary formation is decreased by α-, γ- and δ-tocopherols. Images of HMEC-1 cells on Matrigel, treated with 40 μM tocopherols at 24 h. Capillary tube formation was decreased by α-, γ- or δ-tocopherol when compared to control. (A) 24 h control. (B) 24 h α, 40 μM. (C) 24 h δ, 40 μM. (D) 24 h γ, 40 μM.

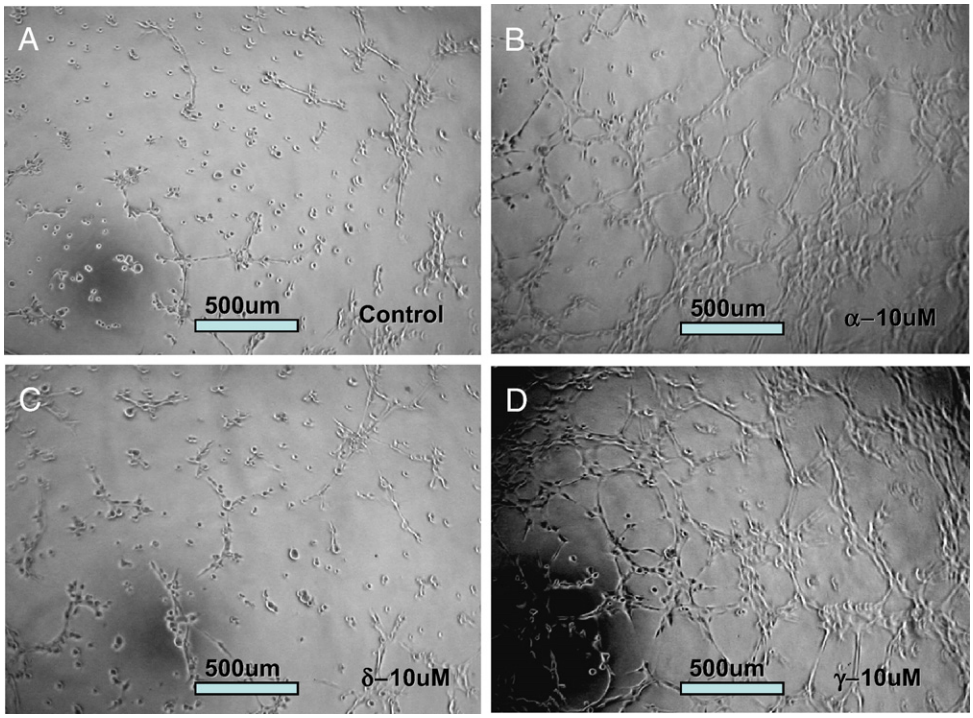

Fig. 3. HMEC-1A (lymphatic endothelial) capillary formation is increased by α- and γ-tocopherols. Images of HMEC-1A cells, grown on Matrigel, treated with 10 μM tocopherols at 24 h. Capillary tube formation increased in α- and γ-tocopherols compared to control. There was no significant change observed in δ-tocopherol when compared to control. (A) 24 h control. (B) 24 h α, 10 μM. (C) 24 h δ, 10 μM. (D) 24 h γ, 10 μM.

3.4. Tocopherols decrease HMEC and lymphatic endothelial cell invasiveness

In HMEC-1, only δ-tocopherol significantly reduced invasiveness, with no effect of δ-tocopherol at 10 or 20 μM; 40 μM δ-tocopherol reduced invasiveness to 57.8±14.6% of control levels (Fig. 4). In lymphatic endothelial cells, α-tocopherol at 40 μM (but not at 10 or 20 μM) significantly reduced invasiveness (to 48.9±8.9%) of control levels seen in Fig. 4. All concentrations of γ-tocopherols (10, 20 and 40 μM) significantly reduced invasiveness (γ10: 26.7±8.7%; γ20: 21.4±8.8%; γ40: 34.1±13.7% of control).

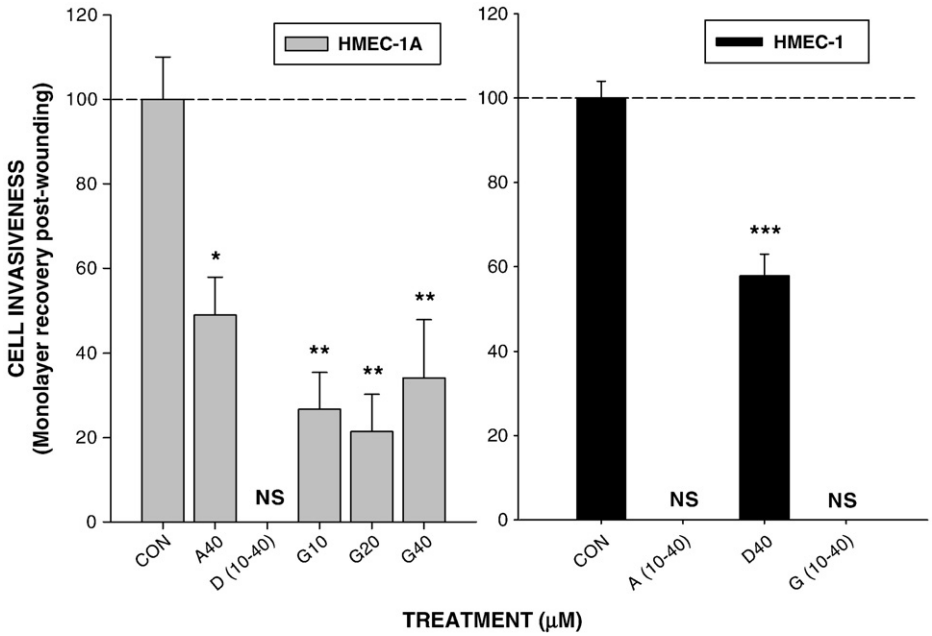

Fig. 4. Effects of tocopherols on blood and lymphatic endothelial invasiveness. Invasiveness of HMEC-1 and HMEC-1A cells treated with different molarities of α-, γ- and δ-tocopherols was compared to untreated controls at 72 h. Results are expressed as a fraction of complete (100%) cell layer recovery±S.E.M. (error bars =S.E.M.). NS, not statistically different from control, bars not shown; *P<.05, significantly different from control; **P<.01, significantly different from control; ***P<.001, significantly different from control using one-way ANOVA, with Dunnett's multiple comparison.

3.5. Tocopherol blockade of TNF-α-induced VCAM-1 expression

TNF-α stimulation (20 ng/ml) for 16 h significantly increased the expression of VCAM-1 in blood vessel endothelium HMEC-1. TNF-α at 20 ng/ml concentration was used as the lowest dose inducing maximal expression of VCAM-1 in the HMEC line. By comparison, HMEC-1, which had been pretreated for 48 h with α-, δ- or γ-tocopherol, showed a dose-dependent reduction in VCAM-1 expression. At 20 and 40 μM, all three tocopherols each significantly reduced TNF-α-induced expression of VCAM-1. α-Tocopherol at 20 and 40 μM reduced VCAM-1 expression by 42% and 53%, respectively (Fig. 5A). γ-Tocopherol reduced VCAM-1 expression at 20 μM by 52% and at 40 μM by 70% (Fig. 5C), while δ-tocopherol reduced VCAM-1 expression by 38% at 20 μM and by 84% at 40 μM (Fig. 5B). None of these tocopherols decreased VCAM expression at 10 μM. In studies using shorter (24 h) tocopherol exposure times, we found that 40 μM γ-tocopherol significantly reduced TNF-α-induced VCAM-1. However, lower concentrations (10–20 μM) of γ-tocopherol and other doses of other tocopherols did not protect against VCAM-1 induction at 24 h. In contrast, lymphatic endothelial cells pretreated with equivalent levels (10–40 μM) of tocopherols did not exhibit a reduction in VCAM expression when exposed to TNF-α (16 h) (Fig. 5D).

3.6. Tocopherols reduced endothelial barrier function in HMEC-1 and HMEC-1A cells

Endothelial barrier function was measured in HMEC-1 and HMEC-1A cells using TEER over a period of 72 h. In HMEC-1, δ-tocopherol was shown to be statistically significant after 24, 48 and 72 h (Con at 24 h, 107.2±12.2; δ40 at 24 h, 81.1±6.1; Con at 48 h, 139.0±10.7; δ40 at 48 h, 61.0±8.1; Con at 72 h, 147.4±16.3; δ40 at 72 h, 65.7±12.2), while α10, γ10 and γ20 were statistically significant in reducing barrier function at 48 h (α10, 122.2±3.3; γ10, 118.5±3.4; γ20, 123.1 ±3.2) (Fig. 6). δ-Tocopherol significantly reduced barrier function at 48 and 72 h (δ20 at 48 h, 90.1±22.3; δ20 at 72 h, 113.5±15.9). In HMEC-1A cells, δ- and γ-tocopherol reduced barrier function at 48 h (Con, 88.9±10.4; δ40, 67.3±8.5; γ10, 72.3±6.3; γ40, 72.4±9.0) only as seen in Fig. 6. α-Tocopherol had no statistically significant effect on endothelial barrier function for lymphatic cells.

4. Discussion

Tocopherols exhibit complex biological effects reflecting their diverse nutritional, antioxidant and signaling properties [23]. The biologically active range of tocopherols is relatively narrow (~3–4-fold variation). Because of this relatively narrow "window of efficacy," benefits from tocopherols might not require consumption of "mega-doses" to be effective; toxicity from overconsumption is uncommon. One reason for a lack of toxicity in antioxidant nutrient supplementation is that the absorption of these compounds in the gastrointestinal tract is a saturable process exhibiting diminishing returns. For example, low doses of vitamin C are nearly completely absorbed, but at higher doses, absorption falls to ~16%. Similarly, ~20–40% of vitamin E (α-tocopherol) is absorbed at the recommended doses with the fractional absorption decreasing with increasing doses. Once ingested, orally administered vitamin E plateaus in the plasma by 24 h but does not fully distribute to all biological compartments until at least ~1 week after ingestion, consistent with its described long half-life [24]. Toxicity and biological activity of tocopherols depend on the species in question, the cell type, chemical vitamer and dosage but

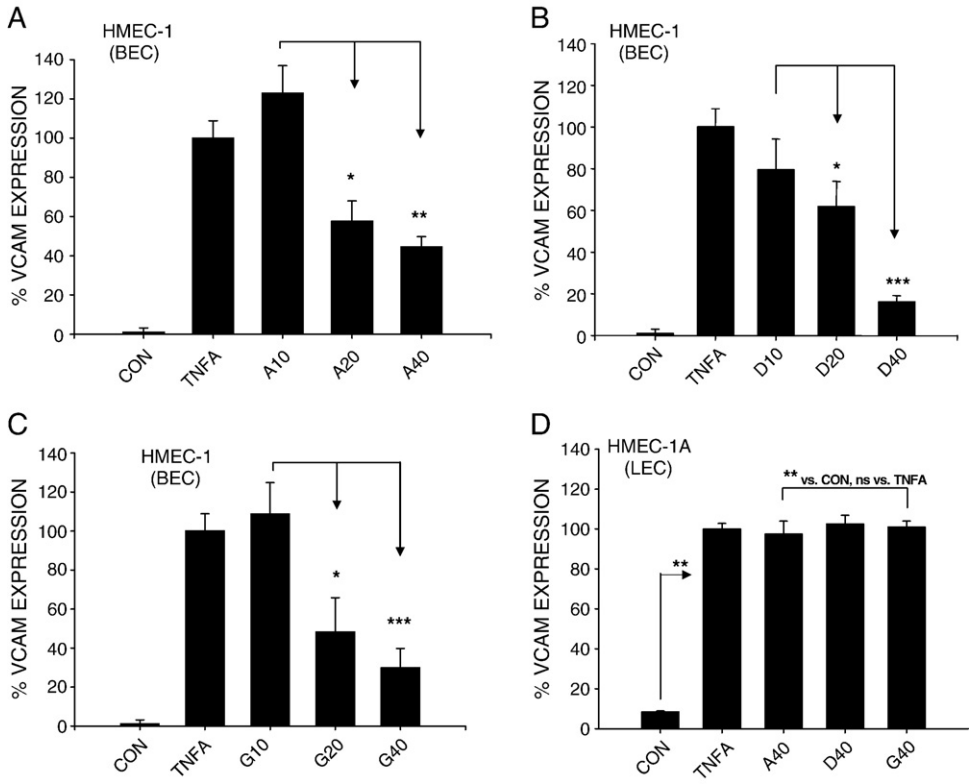

Fig. 5. Tocopherols reduce VCAM expression in HMEC-1 (BEC) but not HMEC-1A (LEC). VCAM expression in HMEC-1 and HMEC-1A cells treated with different molarities of α-, γ- and δ-tocopherols and stimulated with TNFA for 16 h. (A) TNF-α-induced VCAM is decreased by 20 and 40 μM α-tocopherol. (B) TNF-α-induced VCAM is decreased by 20 and 40 μM δ-tocopherol. (C) TNF-α-induced VCAM is decreased by 20 and 40 μM γ-tocopherol. (D) TNF-α-induced VCAM is not decreased by 40 μM α-, γ- or δ-tocopherol in HMEC-1A (lymphatic) cells. % VCAM expression is the fraction of TNF-α-induced VCAM1 (set as 100%) (error bars=S.E.M.). *P<.05, significantly different from TNF-α; **P<.01, significantly different from TNF-α; ***P<.001, significantly different from TNF-α, using one-way ANOVA and Bonferroni posttesting.

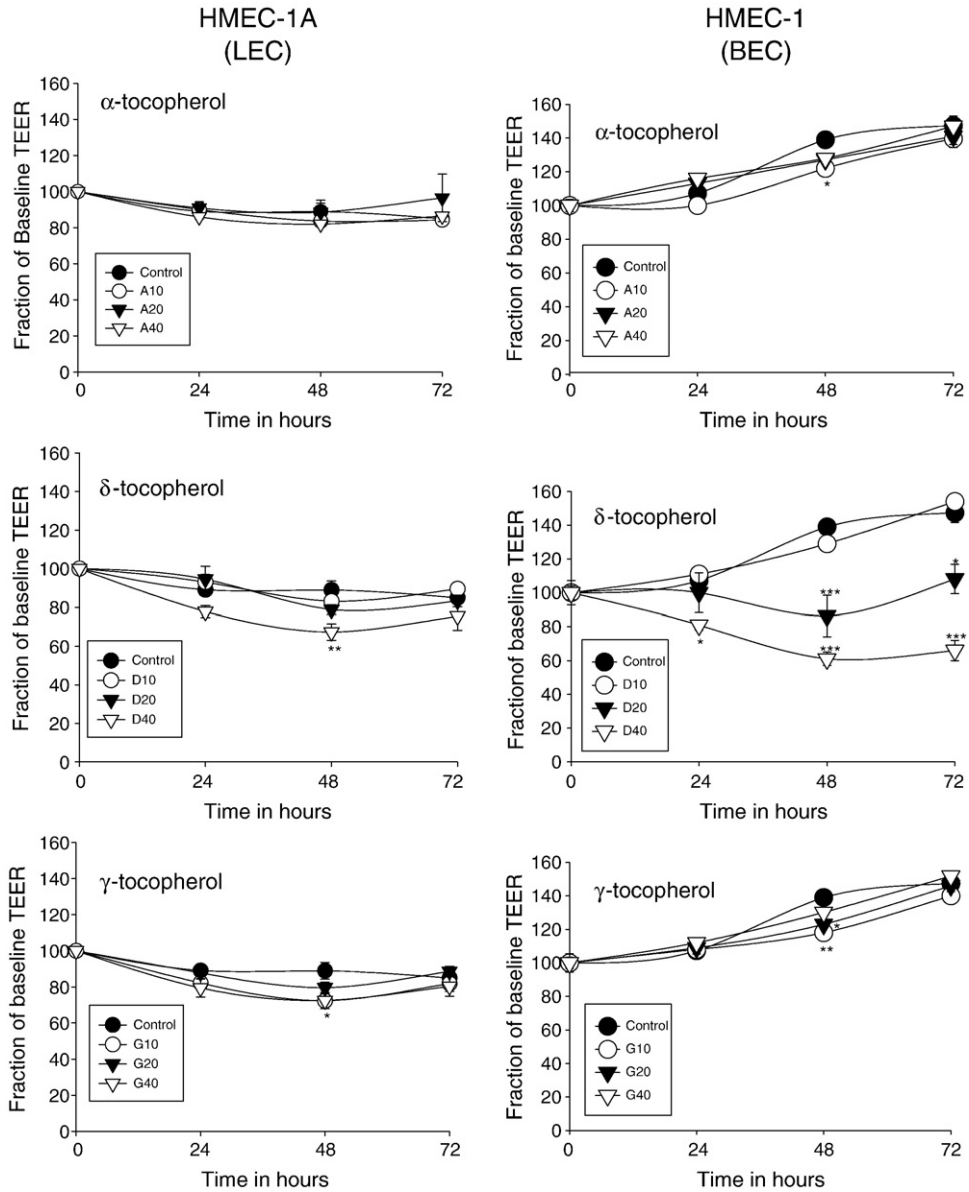

Fig. 6. Effects of tocopherols on TEER. TEER of HMEC-1 (right) and HMEC-1A (left) monolayers was measured after treatment with different molarities (0–40 μM) of α-tocopherol (A10-40), γ-tocopherol (G10-40) and δ-tocopherol (D10-40). TEER was compared to control at 24, 48 and 72 h after addition of tocopherols. Results are expressed as the fraction of baseline TEER (±S.E.M.=error bars). *P<.05, significantly different from control; **P<.01, significantly different from control; ***P<.001, significantly different from control, using one-way ANOVA, Dunnett's multiple comparison.

could be important for evaluating cell responses. For instance, when sufficiently stressed or lysed, cell expression of inflammatory markers like ICAM-1 or VCAM-1 are blocked and could mistakenly be interpreted as "protection" unless accurate viability studies are also carried out.

Biologically "protective" concentrations of tocopherols vary within less than a 10-fold range in plasma. Tocopherol concentrations increase with age and are lower in men than in women [25]. Ford et al. [25] also found the mean human serum concentration of α-tocopherol to be 30.1±0.45 μM and of γ-tocopherol to be 5.7±0.2 μM (US Caucasians) and that plasma tocopherol concentrations vary with demographics. They define vitamin E deficiency (for α-tocopherol) as a plasma concentration at or below 11.6 μM. Symptoms of vitamin E deficiency have been described in individuals with a genetic deficiency in α-tocopherol transfer protein as dying of sensory neurons that causes peripheral neuropathy and ataxia in humans; infertility as well as delayed onset ataxia has been observed in animals [26,27]. African- and Mexican-Americans have been reported to have significantly lower serum concentrations of α-tocopherol than Caucasians (Caucasian, 28.3 μM; African-Americans, 22.6 μM; Mexican-Americans, 24.4 μM). That study concluded that low tocopherol levels in these populations represents nutritional and prooxidative stress which potentially predisposes these groups to greater risks from oxidant linked diseases, gastrointestinal malignancy, cardiovascular and inflammatory diseases (e.g., arthritis, atherosclerosis, Crohn's and ulcerative colitis).

While relatively nontoxic in vivo and in vitro studies on tocopherol-mediated protection against inflammation have sometimes been confounded by an inability to distinguish toxicity of tocopherols from a failure to activate inflammatory pathways, as well as the effects of solvents and carriers, cytotoxic effects of tocopherols also depend on the cell-type and animal species. For example, mouse

macrophages are susceptible to 40 μM tocopherols (particularly γ- and δ-tocopherols), whereas human cells only show cytotoxicity beginning at 90 μM [21]. The concentrations of α- and γ-tocopherols in mouse plasma are 1.3–1.7 and 7.2–13.0 μM, respectively, while the concentrations of tocopherols in human plasma are 2–7 μM for γ- and 15–20 μM for α-tocopherol [28]. Human plasma concentrations of δ-tocopherol were reported to be 70±6 nM [29].

In our current study, tocopherols reduced the proliferation, migration and VCAM-1 expression in blood endothelial cells; therefore, some dietary tocopherols may be beneficial in controlling inflammatory or possibly tumor angiogenesis [30–32]. Interestingly, we find that lymphatic-derived endothelial cells exhibit heterogeneous responses to tocopherols (compared to blood endothelial cells), possibly reflecting different physiological roles and reactions in these conditions.

The relative contributions of the different vitamers are another important consideration, as are biologic interactions between multiple vitamers which are not considered here. For example, α-tocopherol supplementation reduces serum γ- and δ-tocopherol concentrations, possibly modifying or reducing potential benefits that γ- and δ-tocopherols might exert [33]. Despite evidence that α-tocopherol can decrease levels of γ- and δ-tocopherols, α-tocopherol administration at doses equal to or greater than 400 IU/day reduces Cu^{2+}-catalyzed LDL oxidation [34]. Therefore, sufficient levels of an individual tocopherol could protect against oxidant injury.

4.1. Cell proliferation

In vitro, endothelial cells proliferate in response to growth factors, under extracellular matrix cues and in response to several hormonal and environmental factors. HMEC cells exposed to different types and concentration of tocopherols achieved different final confluent densities. γ- and δ-, but not α-tocopherol, significantly reduced confluent cell density of endothelial monolayers. These data indicate that γ- and δ-tocopherols suppress endothelial proliferation leading to an earlier arrest of cell division and lower final cell density (cells/cm^2). Therefore, anti-angiogenic effects of γ- and δ-tocopherols suggest that even modest elevations of these vitamers may limit basal angiogenesis and protect against the greatly increased angiogenesis encountered in cancer and inflammation.

4.2. Effects of tocopherols on capillary formation

It is still controversial whether and which tocopherols modulate capillary angiogenesis.

We found that α-, γ- and δ-tocopherols each reduced several measures of HMEC-1 capillary tube formation on Matrigel in a concentration-dependent manner. Using human umbilical vein endothelial cells, Navarra et al. [35] found α-tocopherol significantly inhibited the formation of intracellular oxidants induced by TNF-α or by phorbol ester (PMA). α-Tocopherol did not reduce the expression of VE-cadherin, α_2-integrin, MMP-1, MMP-2 and MMP-9 (in response to endothelial markers associated with angiogenesis). α-Tocopherol did not affect cell migration or capillary-like tube formation, and at 20 and 40 μM was seen to potentiate PMA-induced DNA synthesis.

Antioxidants inhibit angiogenesis depending on the particular setting and cell type in question. For example, ascorbate blocked the growth inhibition of endothelial cells produced by TNF-α but enhanced endothelial proliferation alone without TNF-α [3].

Many dietary antioxidants have properties that allow for the inactivation of NF-κB gene products through different mechanisms. γ-Tocotrienol promoted apoptosis, decreased cell proliferation and down-regulated VEGF via NF-κB [6]. α-Tocopherol was also effective in reducing VEGF release [20].

Erl et al. [5] have demonstrated that α-tocopherol inhibits monocytic cell adhesion to endothelial cells by suppressing mobilization of the transcription factor NF-κB. γ-Tocotrienol completely stopped TNF-α-induced NF-κB activation while γ-tocopherol had little effect [6]. We previously reported that other antioxidants (melatonin, *N*-acetyl cysteine) can also reduce the endothelial expression of adhesion molecules in response to proinflammatory cytokines [36].

At physiological concentrations, α-tocopherol prevents oxidant-mediated injury and also independently protects cells by the suppression of inflammatory and angiogenic genes [23]. α-Tocopherol also exhibits several biological signaling effects not directly related to antioxidant effects, for example, PKC [23,37].

4.3. Invasiveness

δ-Tocopherol (40 μM) significantly reduced blood vascular endothelial invasiveness, while no significant effect was seen with α- or γ-tocopherol. While δ-tocopherol did not significantly alter lymphatic endothelial invasiveness, high-dose α-tocopherol and all doses of γ-tocopherol reduced invasiveness [38,39], suggesting there may be differential responses of blood and lymphatic endothelial cells to different tocopherols.

4.4. Adhesion molecule expression

TNF-α induces the expression of blood and lymphatic endothelial VCAM-1, a major leukocyte adhesive determinant in inflammation with roles in inflammatory angiogenesis. VCAM-1, an IgCAM expressed on the surface of cytokine or LPS-activated endothelial cells, supports the binding of α4β1 expressing lymphocytes and is a major adhesive ligand in the induction of IBD [40]. We found that α-, γ- and δ-tocopherols each dose-dependently reduced the expression of blood vessel VCAM-1 in response to TNF-α but not in lymphatic endothelial cells.

There are relatively few reports on the biological activity of δ-tocopherol. Reiter et al. [41] found that δ-tocopherol more strongly suppressed cancer cell signaling and proliferation than other tocopherol isoforms. We also find that δ-tocopherol has stronger effects on invasiveness, proliferation and capillary angiogenesis compared to equivalent concentrations of α- and δ-tocopherols while not producing cytotoxicity. δ- and γ-tocopherol show approximately equal potency against TNF-α-induced adhesion molecule expression. Therefore, this is one of the first studies documenting the anti-angiogenic effects of δ-tocopherol on endothelium, indicating that δ-tocopherol might be useful therapeutically. Additional studies will need to be performed which will confirm which specific features are affected by δ-tocopherol. This study also apparently documents lower responsiveness of lymphatic endothelial to tocopherols (compared to blood endothelial cells).

Angiogenesis and lymphangiogenesis play important but different roles in tumorigenesis and inflammation. For example, tumors actively secrete growth factors, for example, VEGFs which recruit new blood and also lymphatic vessels [42–44]. VEGFs also contribute to an overall inflamed phenotype in blood endothelial cells by promoting ICAM-1-dependent leukocyte adhesion [13]. Inflammatory cytokines (e.g., TNF-α) also induce endothelial adhesion molecules (ICAM-1, VCAM-1 and E-selectin) on blood vascular and lymphatic endothelial cells [45,46]. While the expression of ECAMs on blood and lymphatic endothelial cells may both be induced by the same types of stimuli, they may have opposing effects on inflammation. The expression of ECAMs in postcapillary venules is known to mediate the rolling, adhesion and extravasation of immune cells into inflamed tissues (reviewed in Ref. [47]).

Conversely, the induction of ECAMs on lymphatic endothelial cells has been convincingly shown to mediate the exit of immune cells from inflamed tissues [45]. In a model of oxazolone-induced skin inflammation, Johnson et al. [45] showed that the clearance of dendritic cells from the skin was mediated by ICAM-1 and VCAM-1 and that antibody blocking of these ECAMs prevented the exit of immune cells from inflamed tissues.

In this setting, it is interesting to speculate that the persistent activation of NF-κB in lymphatic endothelial cells (but not blood vascular endothelial cells) [48] may explain the observed lymphatic insensitivity of lymphatics to tocopherol blocking of ECAM induction. That is to say α-, γ- and δ- tocopherols may limit inflammation by decreasing ECAM expression in blood vascular endothelium, while lymphatic endothelial cells, refractory to effects of tocopherols on NF-κB, maintain lymphatic ECAMs which allow the exit of these inflammatory immune cells. Berdnikovs et al. [49] have also recently reported in experimental asthma models, combining α- and γ-tocopherols at different ratios may create phenotypes which are protected, or unprotected towards inflammation. This may guide future studies that consider using different tocopherol combinations in the therapeutic reduction (or induction) of inflammatory angiogenesis.

5. Conclusion

Inflammatory angiogenesis, an important event in tissue injury, reflects a hyperproliferative, hyperactivated and proadhesive endothelial phenotype [14]. Oxidants stimulate angiogenesis while antioxidants counteract angiogenesis [50]. Agents like tocopherols which reduce these characteristics are potentially therapeutic.

Since nontoxic levels of tocopherols reduce the proliferation of capillary endothelial cells, they may also control inflammatory angiogenesis, now recognized as an important event in inflammation, and therefore tocopherol attenuation of inflammatory gene activation may control several injury processes in forms of inflammation.

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