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α -Tocopherol Supplementation Reduces 5-Nitro- γ -Tocopherol Accumulation By Decreasing γ -Tocopherol In Young Adult Smokers¹

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Abstract

γ -Tocopherol (γ -T) scavenges reactive nitrogen species (RNS) to form 5-NO₂- γ -tocopherol (NGT). However, α -T supplementation decreases circulating γ -T, which could limit its RNS scavenging activities. We hypothesized that α -T supplementation would mitigate NGT accumulation by impairing γ -T status. Healthy smokers (21 $\pm$ 1 y, n=11) and non-smokers (21 $\pm$ 2 y, n=10) ingested 75 mg/d each of *RRR*- and *all rac*- α -tocopheryl acetate for 6 d. Plasma α -T, γ -T, γ -carboxyethyl-hydroxychromanol (CEHC), NGT, and nitrate/nitrite were measured prior to supplementation (Pre), the morning after 6 consecutive evenings of supplementation (Post 1), and on the mornings of d 6 (Post 6) and d 14 (Post 14) during the post-supplementation period. α -T supplementation increased plasma α -T, and decreased γ -T, in both groups and these returned to Pre concentrations on Post 6 regardless of smoking status. Plasma γ -CEHC increased after the first dose of supplementation in both groups, suggesting that α -T supplementation decreased plasma γ -T in part by increasing its metabolism. Plasma NGT and nitrate/nitrite at Pre were greater in smokers, indicating greater nitrative stress due to cigarette smoking. Plasma NGT was lowered only in smokers on Post 1 and Post 6 and was restored to Pre levels on Post 14. Plasma nitrate/nitrite tended ($P=0.07$) to increase post-supplementation only in smokers, supporting decreases in RNS scavenging by γ -T. Plasma NGT was more strongly correlated ($P<0.05$) with γ -T in smokers ($R=0.83$) compared to non-smokers ($R=0.50$), supporting that α -T-mediated decreases in γ -T reduces NGT formation. These data indicate that α -T supplementation limits γ -T scavenging of RNS in smokers by decreasing γ -T availability.

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Keywords

Reactive nitrogen species; vitamin E; nitro- γ -tocopherol

INTRODUCTION

Reactive nitrogen species (RNS) are a family of compounds derived from nitric oxide ($\text{NO}\bullet$) [1], an important cellular mediator that can be produced by $\text{NO}\bullet$ synthase (NOS) [1]. Relatively low concentrations of $\text{NO}\bullet$, such as those produced constitutively by endothelial NOS (eNOS), regulate various physiological processes including vascular tone and neuronal function [2]. In contrast, pathologic conditions increase NOS-mediated accumulation of $\text{NO}\bullet$, primarily by increasing the expression and activity of inducible NOS (iNOS; [3]). $\text{NO}\bullet$, and its derivatives resulting from reactions with reactive oxygen species (ROS), are collectively known as reactive nitrogen species (RNS). The RNS peroxynitrite, which is the product of the near diffusion-limited reaction between $\text{NO}\bullet$ and superoxide [4], readily reacts with various biomolecules (e.g. lipids, amino acids, and DNA) to disrupt physiological processes [5]. Indeed, increased RNS is associated with various chronic diseases and their risk factors [6].

Unlike α -tocopherol (α -T), γ -T traps RNS including nitrogen dioxide and peroxynitrite to form a stable product, 5- NO_2 - γ -tocopherol (NGT) [7-9]. Studies *in vitro* studies demonstrated that NGT formation occurs directly through the nitration of the free C-5 position on the chromanol head of γ -T or following oxidation of a 5-nitroso- γ -T ortho-quinone monoxime tautomer intermediate [10]. Co-incubation of phosphatidylcholine liposomes with γ -T and a chemical generator of superoxide and $\text{NO}\bullet$ demonstrated that γ -T reduces peroxynitrite-mediated lipid peroxidation, by trapping peroxynitrite as evidenced by the formation of NGT [7]. These observations are consistent with greater NGT concentrations in individuals with increased oxidative and nitrative stress such as coronary disease patients [11], cigarette smokers [12], and Alzheimer's disease patients [13], supporting NGT as a biomarker of nitrative stress. Furthermore, because nitration of γ -T by peroxynitrite occurs more readily than that of tyrosine residues [14], dietary γ -T may be of physiologic importance in limiting cellular nitrative damage.

Despite accumulating evidence from clinical and epidemiological studies suggesting that γ -T may lower the risk of chronic diseases associated with inflammation [15-18], most dietary intervention studies investigating health benefits of vitamin E have focused primarily on α -T, the most abundant form of vitamin E *in vivo*. Findings from these large-scale studies are equivocal in showing that α -T supplementation prevents or treats chronic diseases [19-21]. Furthermore, the hepatic α -tocopherol transfer protein (α -TTP) preferentially directs hepatic α -T for secretion [22]. This limits hepatic secretion of γ -T and results in greater P450-dependent metabolism of γ -T to γ -carboxyethyl-hydroxychroman (γ -CEHC), which is then directed for urinary and biliary elimination [22, 23]. γ -CEHC has anti-inflammatory activities [18], and it has been shown to scavenge RNS [24]. This suggests that metabolism of γ -T to γ -CEHC in response to excess hepatic α -T would limit scavenging of RNS by γ -T.

However, the extent to which increases in γ -T metabolism following α -T supplementation affect scavenging of RNS has never been studied.

We demonstrated that dietary α -T supplementation (150 mg/d of 1:1 mixture of *RRR*- and *all rac*- α -tocopheryl acetates) for 6 d significantly decreased plasma γ -T in both smokers and non-smokers [25], but the extent to which α -T supplementation affected homeostasis of NGT and RNS was not investigated. The current study extends this prior work [25] by examining the hypothesis that α -T supplementation increases γ -T metabolism to γ -CEHC, thus decreasing RNS scavenging by γ -T in cigarette smokers, a free-living clinical model of increased nitrate stress. We examined plasma α - and γ -T, γ -CEHC, NGT, and nitrate/nitrite (NO_x) prior to (Pre) and for 14 d following 6 d of α -T supplementation in smokers and non-smokers.

MATERIALS AND METHODS

Study Participants and Experimental Design

This study was approved by the Institutional Review Board at Oregon State University, and all participants provided written consent before enrollment. Complete details of the study design and intervention have been reported in our work showing that plasma disappearance of α -T is faster in smokers due to their greater oxidative stress [25]. In brief, otherwise healthy smokers (10 cigarettes/d, M/F = 7/4) and non-smokers (M/F = 5/5) were enrolled on the basis of age (18–35 y), normolipidemia, non-nutritional supplement use (6 mo), and exercise status (5 h aerobic activity/wk). Urinary cotinine, the major metabolite of nicotine, was measured by radioimmunoassay (Diagnostics Products Corp, Los Angeles, CA, USA). Urinary cotinine concentrations >500 ng/mL were used to confirm smoking status as suggested by the manufacturer.

On 6 consecutive evenings, participants ingested an encapsulated vitamin E supplement containing 150 mg deuterium labeled α -T (75 mg/d each of d_3 -*RRR*- and d_6 -*all-rac*- α -tocopheryl acetate) with a standardized meal consisting of 1143 kcal (43% carbohydrate, 17% protein, and 41% fat), 35 mg ascorbic acid, and 2.7 mg α -T [25]. Administration of *RRR*- and *all-rac*- α -tocopheryl acetate reflected that the American diet contains both natural and synthetic forms of α -T while also allowing for differential examination of their pharmacokinetics in smokers and non-smokers as we reported previously [25]. Prior to any supplementation, and in the morning following an overnight fast, participants visited the study center for the collection of a baseline blood sample (Pre). They then initiated the supplementation regimen that evening for 6 consecutive evenings. For the present study, blood samples were limited to those obtained prior to receiving any α -T supplementation (Pre), the morning after receiving supplementation for 6 consecutive evenings (Post 1), and on the mornings of d 6 (Post 6) and d 14 (Post 14) during the post-supplementation period (Figure 1). Plasma γ -CEHC concentrations were published previously [26], but are reported herein to illustrate the relationship of α -T supplementation on γ -T metabolism. For the present study, we report circulating γ -CEHC concentrations at Pre, the morning after receiving the first α -tocopherol supplement ($1_{\alpha\text{-T}}$), the morning after 3 consecutive days of supplementation ($3_{\alpha\text{-T}}$), and at Post 1 and Post 6. Blood was collected from the antecubital vein and processed as described [25]. Plasma was aliquoted into cryovials, snap-frozen in

liquid nitrogen, and archived at -80°C . Smokers were instructed to refrain from smoking for 1 h before blood collection to alleviate transient oxidative/nitrative stress effects.

Materials

HPLC-grade solvents, ascorbic acid, potassium hydroxide (KOH) and butylated hydroxytoluene were obtained from Fisher (Fair Lawn, NJ, USA). dl-Tocol (internal standard, purity $>95\%$) was purchased from Matreya LLC (Pleasant Gap, PA, USA) and α -tocopheryl acetate was purchased from Sigma (St. Louis, MO, USA). NGT is not available commercially and was synthesized from γ -T as described [7]. Briefly, an ethanolic solution of γ -T was acidified with glacial acetic acid and nitration was subsequently induced by sodium nitrite. After terminating the reaction with KOH, and washing the mixture with water, the crude product was extracted using hexane, dried under nitrogen gas and reconstituted in ethanol. Purity of NGT was $>99\%$ as determined by LC-MS and its concentration was determined spectrophotometrically (Shimadzu UV-2600, Columbia, MD, USA) in ethanol using $\epsilon_{410\text{ nm}} = 1976\text{ M}^{-1}\text{cm}^{-1}$ [12].

Analyses

α -T, γ -T, and NGT were extracted as described [27], with minor modifications. In brief, plasma was saponified with alcoholic KOH and subsequently extracted with hexane. Samples were evaporated under nitrogen gas and redissolved in 1:1 methanol:ethanol for analysis using a Prominence UFLC-MS system (Shimadzu, Columbia, MD, USA) consisting of an autosampler maintained at 4°C (SIL-30AC), a degassing unit (DGU-20A5), a column oven set to 30°C (CTO-30 A), two LC-30AD pumps, and an LC-MS-2020 single-quadrupole mass spectrometer equipped with a dual-ionization source operated in negative mode. Instrument control was performed using Shimadzu LabSolution (version 5.4). Analytes were separated isocratically using 100% methanol on a Kinetex C18 column ($100 \times 2.1\text{ mm}$, $2.6\text{ }\mu\text{m}$; Phenomenex, Torrance, CA, USA) and detected using single ion recording at the following mass-to-charge (m/z) ratios: unlabeled (d_0)- α -T, m/z 429.4, d_3 - α -T, m/z 432.4, d_6 - α -T, m/z 435.4, γ -T, m/z 415.4, NGT, m/z 460.4, and dl-tocol (internal standard), m/z 387.4. For the present study, analytes were quantified using external standards relative to dl-tocol (internal standard), and α -T data were reported as total α -T (i.e. sum of d_0 -, d_3 -, and d_6 - α -T), which was used in all analyses to define α -T supplementation on changes in NGT. α - and γ -CEHC were analyzed from plasma and reported previously [26]. Plasma total nitrite/nitrate (NO_x) was measured as an index of $\text{NO}\bullet$ status using a colorimetric spectrophotometric kit (Sigma, St. Louis, MO, USA).

Statistical analysis

Data (means $\pm$ SEM) were analyzed using SPSS 17.0 (Chicago, IL, USA). An unpaired Student's t-test was used for all comparisons between smokers and non-smokers at Pre. Twoway repeated measures ANOVA was used to evaluate main effects due to smoking and time, and smoking by time interactions followed by Bonferroni post hoc tests to examine pair-wise differences. To examine the association between plasma NGT and γ -T, multiple linear regression controlling for repeated measures was conducted [28]. Data were considered statistically significant at P 0.05.

RESULTS

Participants

As reported previously [25], participants were generally healthy with all clinical chemistries within normal clinical limits. There were no significant differences ($P>0.05$) in age, body mass index, circulating enzyme markers, plasma lipids, α -T, γ -T, α -CEHC, uric acid, ascorbic acid at Pre between smokers and non-smokers (**Table 1**). Serum alkaline phosphatase tended ($P=0.10$) to be greater in smokers vs. non-smokers, but was still within normal clinical limits. Self-reported smoking frequency was 10-20 cigarettes/d and urinary cotinine was >500 ng/mL for all smokers. In contrast, non-smokers had low (<78 ng/mL) urinary cotinine concentrations indicating their non-smoking status and limited exposure to second-hand smoke.

Tocopherols and CEHC

Plasma α - and γ -T concentrations were not different between smokers and non-smokers prior to initiating 6 d of supplementation (Pre; **Table 1**). After 6 d of α -T supplementation (Post 1), plasma total α -T increased by 41-50% ($P<0.05$) with no difference between groups (**Figure 2A**). Plasma total α -T in both groups returned to concentrations no different from Pre by d 6 post-supplementation (Post 6). Regardless of smoking status, on Post 1 (the morning following 6 d of α -T supplementation) plasma γ -T concentrations were 61-70% lower compared to Pre (**Figure 2B**). Plasma γ -T returned to concentrations no different from Pre at Post 6 of α -T.

To better define decreases in plasma γ -T in response to α -T supplementation, we examined plasma γ -CEHC to illustrate α -tocopherol-mediated changes in γ -T metabolism. At Pre, plasma γ -CEHC was 47% lower in smokers compared to non-smokers (**Table 1**). However, it increased significantly in both smokers and non-smokers at 1 α -T (i.e. the morning following the first dose of α -T supplementation) and returned to levels no different from Pre at 3 α -T (**Figure 3**). This study was originally designed to examine the pharmacokinetics and metabolism of α -T following supplementation of deuterium-labeled α -T in smokers and non-smokers [26]. Plasma CEHC concentrations were only measured up to Post 6 because deuterium-labeled α -CEHC was undetectable at later time points [26].

5-NO₂- γ -Tocopherol

Smokers' concentrations of plasma NGT at Pre were approximately doubled those of non-smokers (**Table 1**), consistent with smokers having greater nitrate stress [29]. We hypothesized that α -T supplementation-mediated increases in γ -T metabolism would lower NGT, suggestive of decreases in RNS scavenging activity by γ -T. A significant smoking by time interaction indicated that non-smokers' NGT concentrations were unaffected by α -T supplementation, but that smokers' NGT concentrations were 73% lower on Post 1 and remained lower than Pre levels until Post 6 (**Figure 4A**). The lack of change in non-smokers and significant decrease in plasma NGT in smokers was more apparent when examining changes in plasma NGT concentrations (**Figure 4B**). NGT concentrations of smokers returned to concentrations no different from Pre concentrations at d 14 post-supplementation (Post 14) regardless of examining changes in plasma NGT concentrations (**Figure 4A-B**).

Multiple linear regression analysis indicated that plasma γ -T was correlated to plasma NGT in both non-smokers ($P < 0.05$, $R = 0.50$) and smokers ($P < 0.001$, $R = 0.83$; **Figure 5**). However, the regression slope of γ -T in relation to NGT for smokers was ~2-times greater than that of non-smokers (12.41 vs. 5.67 nmol/ μ mol; $P < 0.05$). This suggests that, for a given concentration of γ -T, a greater extent of it is utilized for RNS scavenging in smokers compared to non-smokers.

Total nitrate/nitrite

Consistent with their greater plasma NGT, smokers had 44% higher concentrations of NO_x compared with non-smokers at Pre (**Table 1**). Plasma NGT concentrations of smokers were also higher than that of non-smokers at Post 1, as α -T supplementation tended to increase NO_x in smokers ($P = 0.065$; **Figure 6**), but not that of non-smokers at Post 1. In contrast, there were no statistical differences between smokers and non-smokers at Post 6 and Post 14.

DISCUSSION

This study demonstrated that NGT accumulation was attenuated in smokers, but not in non-smokers, due to decreased availability of γ -T following α -T supplementation. α -T supplementation increased plasma γ -CEHC the morning after receiving the first dose, supporting that α -T supplementation decreased γ -T by increasing its cytochrome P450-mediated metabolism. Supplementation of α -T also decreased plasma NGT and tended to increase plasma NO_x only in smokers, supporting an attenuation of γ -T mediated scavenging of RNS by α -T supplementation, specifically in a population that is routinely characterized by high RNS [30]. Plasma γ -T was positively correlated with plasma NGT in both groups, although this relationship was stronger in smokers compared to non-smokers. Collectively, these findings suggest that NGT accumulation is dependent on γ -T availability, particularly in smokers with high levels of RNS, and the use of NGT as a nitrative stress biomarker may be limited to physiologic models in which γ -T status is unaffected by the intervention, especially under conditions of chronic nitrative stress.

An estimated 41% of American adults take α -T supplements, with 11% consuming 400 IU/d [31], despite inconclusive evidence for health benefits of supra-physiologic intakes of α -T [18]. A meta-analysis of clinical studies indicates that supplementation of α -T has no effect on all-cause mortality [32] or in preventing specific chronic disease such as cardiovascular disease and cancer [19,21, 33]. Others suggest that supplementation of α -T at 400 IU/d may increase all-cause mortality [34], although this is controversial [35-38]. Regardless, it is well documented that α -T supplementation decreases circulating levels of γ -T [18], a lesser studied form of vitamin E that directly scavenges RNS while also exerting anti-oxidative and anti-inflammatory activities [18]. In support, we showed that α -T supplementation increased γ -CEHC on the morning after the first α -T supplement dose (i.e. 1 $_{\alpha$ -T). This suggests that decreases in plasma γ -T observed at Post 1 are at least partly mediated by increases in cytochrome P450-mediated metabolism of γ -T to γ -CEHC. Supplementation of α -T may also decrease γ -T status by increasing its biliary elimination [39, 40]. This is consistent with studies in rodents showing that injections of supra-

physiologic levels of α -T increased hepatic mRNA expression of the phase III efflux transporters *ABCB1b* and *ABCG2* that are involved in biliary elimination of xenobiotics, such as vitamin E [41]. Additional study in more invasive model systems is needed to define the mechanisms by which α -T supplementation increases γ -T elimination. Regardless of the mechanism, our data clearly demonstrate that short-term α -T supplementation decreased plasma γ -T, consistent with others [12], and enables testing of our hypothesis that scavenging of RNS by γ -T would be reduced.

Unlike α -T, γ -T is able to trap RNS to form NGT due to the unsubstituted 5-position of its chromanol head [7, 8]. Consistent with this, γ -T treatment attenuated protein nitration in murine kidneys following zymosan-induced peritonitis, as indicated by a decrease in kidney nitrotyrosine and a concomitant increase in NGT [42]. Attenuating protein nitration is relevant to the etiology and prevention of chronic diseases, particularly those mediated by inflammation including CVD, rheumatoid arthritis, multiple sclerosis, and Alzheimer's [43]. Although γ -CEHC also has an unsubstituted position on its chromanol head, scavenging of RNS by γ -CEHC has not been documented *in vivo* nor has 5-NO₂- γ -CEHC been detected from biological samples [44]. Our results suggest that increased metabolism of γ -T to γ -CEHC following α -T supplementation decreased RNS scavenging by γ -T. Indeed, α -T supplementation-mediated decreases in γ -T lowered plasma NGT only in smokers, likely because they have greater nitrate stress compared to non-smokers. The decrease in NGT formation following α -T supplementation was accompanied by an increase of NO_x, stable end-products of NO• and its derivatives. The limited specificity of NO_x as a biomarker of RNS is a major limitation of using it to assess nitrate stress. Indeed, NO_x formation results from oxyhemoglobin-mediated metabolism of NO• [45] and also through the decomposition of peroxynitrite [46]. We observed a tendency for NO_x to increase in smokers in the present study. While this suggests an increase in nitrate stress due to an attenuation of RNS scavenging by γ -T, consideration is needed to utilize a more specific and sensitive nitrate stress biomarker in future studies to better assess changes in RNS.

One of the most commonly measured marker of nitrate stress is nitrotyrosine. Nitrotyrosine accumulation occurs in numerous human diseases and inflammatory conditions such as multiple sclerosis, spinal cord injury, stroke and ischemia/reperfusion [47]. Nitration of tyrosine residues results in loss of protein function or cell death and can contribute to disease pathogenesis [47, 48]. Unfortunately, the use of nitrotyrosine as a marker of nitrate stress is limited mainly due to analytical challenges associated with immunological assays, the most convenient and cost-effective method of measuring nitrotyrosine [49, 50]. More rigorous MS-based assays have circumvented these limitations, but artifactual formation of nitrotyrosine occurs during sample preparation if precautions are not taken [51, 52]. Thus, effort is warranted to validate complementary biomarkers of RNS-mediated damage such as NGT. Measuring NGT by LC-MS is rapid, accurate, and sensitive [12]. Increased levels of NGT have been observed in conditions associated with nitrate stress [11-13]. Two- to three-times greater NGT concentrations were found in regions of brain having evidence of Alzheimer's disease [13] and NGT was elevated in plasma and atherosclerotic plaque of coronary heart disease patients [11]. Furthermore, co-incubation of γ -T with peroxynitrite and tyrosine inhibited nitrotyrosine formation in liposomes by up to 90%, suggesting that peroxynitrite reacts more readily with γ -T compared to tyrosine [14].

This supports NGT as a marker of nitrate stress and complements measurements of nitrotyrosine, which would be expected to accumulate more appreciably upon depletion of γ -T.

We previously demonstrated that smokers' plasma γ -T disappearance rates were 2.7-fold faster than their γ -CEHC formation rates, an imbalance that was restored by ascorbic acid supplementation [53], suggesting utilization of γ -T in smokers is due to increased oxidation/nitration. The present study demonstrated a decrease in smokers' plasma NGT accumulation when γ -T concentrations were reduced by α -T supplementation, and that NGT was subsequently restored when plasma γ -T was restored to pre- α -T supplementation levels. Regression analysis also demonstrated a stronger relationship between NGT and γ -T concentrations in smokers compared to non-smokers. This suggests an intimate relationship between NGT formation and γ -T and RNS availability, and supports additional study of NGT as a biomarker of nitrate stress, specifically when γ -T status is carefully monitored.

In conclusion, we demonstrated that α -T supplementation in smokers attenuates γ -T scavenging of RNS by decreasing the availability of γ -T. Additional study is warranted to better define the extent to which α -T supplementation impairs γ -T-dependent scavenging of RNS and whether depletion of γ -T in response to α -T supplementation increases smokers' nitrate stress and their risk for chronic disease. This knowledge is of significant public health importance considering that 22% of Americans smoke [54] and that dietary α -T supplements are frequently used in the U.S. [31].

REFERENCES

1. Li H, Horke S, Forstermann U. Vascular oxidative stress, nitric oxide and atherosclerosis. *Atherosclerosis*. 2014; 237:208–219. [PubMed: 25244505]
2. Ridnour LA, Thomas DD, Mancardi D, Espey MG, Miranda KM, Paolocci N, et al. The chemistry of nitrosative stress induced by nitric oxide and reactive nitrogen oxide species. Putting perspective on stressful biological situations. *Biological Chemistry*. 2004; 385:1–10. [PubMed: 14977040]
3. Seimetz M, Parajuli N, Pichl A, Veit F, Kwapiszewska G, Weisel FC, et al. Inducible NOS inhibition reverses tobacco-smoke-induced emphysema and pulmonary hypertension in mice. *Cell*. 2011; 147:293–305. [PubMed: 22000010]
4. Huie RE, Padmaja S. The reaction of NO with superoxide. *Free Radic Res Commun*. 1993; 18:195–199. [PubMed: 8396550]
5. Patel RP, McAndrew J, Sellak H, White CR, Jo H, Freeman BA, et al. Biological aspects of reactive nitrogen species. *Biochimica et Biophysica Acta - Bioenergetics*. 1999; 1411:385–400.
6. Pacher P, Beckman JS, Liaudet L. Nitric oxide and peroxynitrite in health and disease. *Physiological Reviews*. 2007; 87:315–424. [PubMed: 17237348]
7. Christen S, Woodall AA, Shigenaga MK, Southwell-Keely PT, Duncan MW, Ames BN. γ -Tocopherol traps mutagenic electrophiles such as NOx and complements α -tocopherol: Physiological implications. *Proceedings of the National Academy of Sciences of the United States of America*. 1997; 94:3217–3222. [PubMed: 9096373]
8. Cooney RV, Franke AA, Harwood PJ, Hatch-Pigott V, Custer LJ, Mordan LJ. γ -Tocopherol detoxification of nitrogen dioxide: Superiority to α -tocopherol. *Proceedings of the National Academy of Sciences of the United States of America*. 1993; 90:1771–1775. [PubMed: 8446589]
9. Hoglen NC, Waller SC, Sipes IG, Liebler DC. Reactions of peroxynitrite with γ -tocopherol. *Chemical Research in Toxicology*. 1997; 10:401–407. [PubMed: 9114976]

10. Patel A, Liebner F, Netscher T, Mereiter K, Rosenau T. Vitamin E chemistry. Nitration of non-alpha-tocopherols: products and mechanistic considerations. *J Org Chem.* 2007; 72:6504–6512. [PubMed: 17636958]
11. Morton LW, Ward NC, Croft KD, Puddey IB. Evidence for the nitration of γ -tocopherol in vivo: 5-nitro- γ -tocopherol is elevated in the plasma of subjects with coronary heart disease. *Biochemical Journal.* 2002; 364:625–628. [PubMed: 11960550]
12. Leonard SW, Bruno RS, Paterson E, Schock BC, Atkinson J, Bray TM, et al. 5-nitro- γ -tocopherol increases in human plasma exposed to cigarette smoke in vitro and in vivo. *Free Radical Biology and Medicine.* 2003; 35:1560–1567. [PubMed: 14680679]
13. Williamson KS, Prasad Gabbita S, Mou S, West M, Pye QN, Markesbery WR, et al. The nitration product 5-nitro- γ -tocopherol is increased in the Alzheimer brain. *Nitric Oxide - Biology and Chemistry.* 2002; 6:221–227.
14. Goss SP, Hogg N, Kalyanaraman B. The effect of alpha-tocopherol on the nitration of gamma-tocopherol by peroxynitrite. *Arch Biochem Biophys.* 1999; 363:333–340. [PubMed: 10068456]
15. Kushi LH, Folsom AR, Prineas RJ, Mink PJ, Wu Y, Bostick RM. Dietary antioxidant vitamins and death from coronary heart disease in postmenopausal women. *N Engl J Med.* 1996; 334:1156–1162. [PubMed: 8602181]
16. Kristenson M, Zieden B, Kucinskiene Z, Elinder LS, Bergdahl B, Elwing B, et al. Antioxidant state and mortality from coronary heart disease in Lithuanian and Swedish men: concomitant cross sectional study of men aged 50. *BMJ.* 1997; 314:629–633. [PubMed: 9066473]
17. Kontush A, Spranger T, Reich A, Baum K, Beisiegel U. Lipophilic antioxidants in blood plasma as markers of atherosclerosis: the role of alpha-carotene and gamma-tocopherol. *Atherosclerosis.* 1999; 144:117–122. [PubMed: 10381285]
18. Jiang Q. Natural forms of vitamin E: metabolism, antioxidant, and anti-inflammatory activities and their role in disease prevention and therapy. *Free Radic Biol Med.* 2014; 72C:76–90. [PubMed: 24704972]
19. Myung SK, Ju W, Cho B, Oh SW, Park SM, Koo BK, et al. Efficacy of vitamin and antioxidant supplements in prevention of cardiovascular disease: systematic review and meta-analysis of randomised controlled trials. *BMJ.* 2013; 346:f10. [PubMed: 23335472]
20. Vardi M, Levy NS, Levy AP. Vitamin E in the prevention of cardiovascular disease: the importance of proper patient selection. *J Lipid Res.* 2013; 54:2307–2314. [PubMed: 23505320]
21. Cardenas E, Ghosh R. Vitamin E: a dark horse at the crossroad of cancer management. *Biochem Pharmacol.* 2013; 86:845–852. [PubMed: 23919929]
22. Mustacich DJ, Bruno RS, Traber MG. Vitamin E. *Vitam Horm.* 2007; 76:1–21. [PubMed: 17628169]
23. Mustacich DJ, Shields J, Horton RA, Brown MK, Reed DJ. Biliary secretion of alpha-tocopherol and the role of the mdr2 P-glycoprotein in rats and mice. *Arch Biochem Biophys.* 1998; 350:183–192. [PubMed: 9473291]
24. Galli F, Piroddi M, Lannone A, Pagliarani S, Tomasi A, Floridi A. A comparison between the antioxidant and peroxynitrite-scavenging functions of the vitamin E metabolites alpha- and gamma-carboxyethyl-6-hydroxychromans. *Int J Vitam Nutr Res.* 2004; 74:362–373. [PubMed: 15628675]
25. Bruno RS, Ramakrishnan R, Montine TJ, Bray TM, Traber MG. α -tocopherol disappearance is faster in cigarette smokers and is inversely related to their ascorbic acid status. *American Journal of Clinical Nutrition.* 2005; 81:95–103. [PubMed: 15640466]
26. Bruno RS, Leonard SW, Li J, Bray TM, Traber MG. Lower plasma alpha-carboxyethylhydroxychroman after deuterium-labeled alpha-tocopherol supplementation suggests decreased vitamin E metabolism in smokers. *Am J Clin Nutr.* 2005; 81:1052–1059. [PubMed: 15883428]
27. Mah E, Pei R, Guo Y, Ballard KD, Barker T, Rogers VE, et al. gamma-Tocopherol-rich supplementation additively improves vascular endothelial function during smoking cessation. *Free Radic Biol Med.* 2013; 65:1291–1299. [PubMed: 24075893]
28. Bland JM, Altman DG. Calculating correlation coefficients with repeated observations: Part 1--Correlation within subjects. *BMJ.* 1995; 310:446. [PubMed: 7873953]

29. Bruno RS, Traber MG. Cigarette smoke alters human vitamin E requirements. *J Nutr.* 2005; 135:671–674. [PubMed: 15795416]
30. Colombo G, Clerici M, Giustarini D, Portinaro NM, Aldini G, Rossi R, et al. Pathophysiology of tobacco smoke exposure: recent insights from comparative and redox proteomics. *Mass Spectrom Rev.* 2014; 33:183–218. [PubMed: 24272816]
31. Ford ES, Ajani UA, Mokdad AH. Brief communication: The prevalence of high intake of vitamin E from the use of supplements among U.S. adults. *Ann Intern Med.* 2005; 143:116–120. [PubMed: 16027453]
32. Abner EL, Schmitt FA, Mendiondo MS, Marcum JL, Krysci RJ. Vitamin E and all-cause mortality: A meta-analysis. *Current Aging Science.* 2011; 4:158–170. [PubMed: 21235492]
33. Bin Q, Hu X, Cao Y, Gao F. The role of vitamin E (tocopherol) supplementation in the prevention of stroke: A meta-analysis of 13 randomised controlled trials. *Thrombosis and Haemostasis.* 2011; 105:579–585. [PubMed: 21264448]
34. Miller Iii ER, Pastor-Barriuso R, Dalal D, Riemersma RA, Appel LJ, Guallar E. Meta-analysis: High-dosage vitamin E supplementation may increase all-cause mortality. *Annals of Internal Medicine.* 2005; 142:37–46+I-40. [PubMed: 15537682]
35. Guallar E, Hanley DF, Miller ER 3rd. An editorial update: Annus horribilis for vitamin E. *Ann Intern Med.* 2005; 143:143–145. [PubMed: 16027457]
36. Meydani SN, Lau J, Dallal GE, Meydani M. High-dosage vitamin E supplementation and all-cause mortality. *Ann Intern Med.* 2005; 143:153. author reply 156–158. [PubMed: 16027466]
37. Blatt DH, Pryor WA. High-dosage vitamin E supplementation and all-cause mortality. *Ann Intern Med.* 2005; 143:150–151. author reply 156–158. [PubMed: 16027460]
38. Greenberg ER. Vitamin E supplements: good in theory, but is the theory good? *Ann Intern Med.* 2005; 142:75–76. [PubMed: 15579660]
39. Bardowell SA, Ding X, Parker RS. Disruption of P450-mediated vitamin E hydroxylase activities alters vitamin E status in tocopherol supplemented mice and reveals extra-hepatic vitamin E metabolism. *J Lipid Res.* 2012; 53:2667–2676. [PubMed: 22969154]
40. Traber MG, Kayden HJ. Preferential incorporation of alpha-tocopherol vs gamma-tocopherol in human lipoproteins. *Am J Clin Nutr.* 1989; 49:517–526. [PubMed: 2923084]
41. Traber MG, Labut EM, Leonard SW, Lebold KM. alpha-Tocopherol injections in rats up-regulate hepatic ABC transporters, but not cytochrome P450 enzymes. *Free Radic Biol Med.* 2011; 51:2031–2040. [PubMed: 21945367]
42. Jiang Q, Lykkesfeldt J, Shigenaga MK, Shigeno ET, Christen S, Ames BN. Gamma-tocopherol supplementation inhibits protein nitration and ascorbate oxidation in rats with inflammation. *Free Radic Biol Med.* 2002; 33:1534–1542. [PubMed: 12446211]
43. Greenacre SA, Ischiropoulos H. Tyrosine nitration: localisation, quantification, consequences for protein function and signal transduction. *Free Radic Res.* 2001; 34:541–581. [PubMed: 11697033]
44. Wu JH, Hodgson JM, Ward NC, Clarke MW, Puddey IB, Croft KD. Nitration of gamma-tocopherol prevents its oxidative metabolism by HepG2 cells. *Free Radic Biol Med.* 2005; 39:483–494. [PubMed: 16043020]
45. Kelm M. Nitric oxide metabolism and breakdown. *Biochim Biophys Acta.* 1999; 1411:273–289. [PubMed: 10320663]
46. Szabo C, Ischiropoulos H, Radi R. Peroxynitrite: biochemistry, pathophysiology and development of therapeutics. *Nat Rev Drug Discov.* 2007; 6:662–680. [PubMed: 17667957]
47. Franco MC, Estevez AG. Tyrosine nitration as mediator of cell death. *Cell Mol Life Sci.* 2014; 71:3939–3950. [PubMed: 24947321]
48. DiDonato JA, Aulak K, Huang Y, Wagner M, Gerstenecker G, Topbas C, et al. Site-specific nitration of apolipoprotein A-I at tyrosine 166 is both abundant within human atherosclerotic plaque and dysfunctional. *J Biol Chem.* 2014; 289:10276–10292. [PubMed: 24558038]
49. Tsikas D. Analytical methods for 3-nitrotyrosine quantification in biological samples: the unique role of tandem mass spectrometry. *Amino Acids.* 2012; 42:45–63. [PubMed: 20495837]
50. Safinowski M, Wilhelm B, Reimer T, Weise A, Thome N, Hanel H, et al. Determination of nitrotyrosine concentrations in plasma samples of diabetes mellitus patients by four different

immunoassays leads to contradictory results and disqualifies the majority of the tests. Clin Chem Lab Med. 2009; 47:483–488. [PubMed: 19284298]

51. Tsikas D, Schwedhelm E, Frolich JC. Methodological considerations on the detection of 3-nitrotyrosine in the cardiovascular system. Circ Res. 2002; 90:E70. [PubMed: 11934846]
52. Tsikas D. Measurement of nitrotyrosine in plasma by immunoassays is fraught with danger: commercial availability is no guarantee of analytical reliability. Clin Chem Lab Med. 2010; 48:141–143. author reply 145-146. [PubMed: 20047534]
53. Bruno RS, Leonard SW, Atkinson J, Montine TJ, Ramakrishnan R, Bray TM, et al. Faster plasma vitamin E disappearance in smokers is normalized by vitamin C supplementation. Free Radic Biol Med. 2006; 40:689–697. [PubMed: 16458200]
54. U.S. Department of Health and Human Services. A Report of the Surgeon General. U.S. Department of Health and Human Services, Centers for Disease Control and Prevention, National Center for Chronic Disease Prevention and Health Promotion, Office on Smoking and Health; Atlanta, GA: 2014. The Health Consequences of Smoking: 50 Years of Progress..

Figure 1. Study Design

Non-smokers ($n = 10$) and smokers ($n = 11$) were provided 75 mg/d each of *RRR*- α -tocopheryl acetate and *all rac*- α -tocopheryl acetate for 6 consecutive evenings. For measurements of all biomarkers, blood samples were collected in the morning after an overnight fast prior to receiving any supplementations (Pre), the morning after receiving daily supplementation for 6 consecutive evenings (Post 1), and on the mornings of d 6 (Post 6) and d 14 (Post 14) during the post-supplementation period. For the assessment of γ -tocopherol metabolism, the present study utilizes plasma γ -carboxyethyl-hydroxychromanol data reported previously [26] at Pre, the morning after receiving the first α -tocopherol supplement ($1_{\alpha-T}$), the morning after 3 consecutive evenings of supplementation ($3_{\alpha-T}$), and at Post 6 and Post 14.

Figure 2. Plasma α - and γ -tocopherol concentrations from non-smokers ($n = 10$) and smokers ($n = 11$) prior to and following supplementation with 75 mg/d each of RRR- α -tocopheryl acetate and all rac- α -tocopheryl acetate for 6 consecutive evenings

Plasma tocopherols were measured prior to (Pre), the morning after receiving daily supplementation for 6 consecutive evenings (Post 1), and on the mornings of d 6 (Post 6) and d 14 (Post 14) during the post-supplementation period. Data are means $\pm$ SEM. 2-Way repeated measures ANOVA with Bonferroni correction were performed to evaluate time, smoking, and interaction effects (* $P < 0.05$ as compared with Pre).

Figure 3. Plasma γ -carboxyethyl-hydroxychromanol (γ -CEHC) concentrations from non-smokers ($n = 10$) and smokers ($n = 11$) prior to, during, and following supplementation with 75 mg/d each of RRR- α -tocopheryl acetate and all rac- α -tocopheryl acetate for 6 consecutive evenings

Plasma γ -CEHC was measured prior to receiving any supplementation (Pre), the morning after receiving the first α -tocopherol supplement (1 α -T), the morning after 3 consecutive evenings of supplementation (3 α -T), and on the mornings of d 6 (Post 6) and d 14 (Post 14) during the post-supplementation period. Data are means $\pm$ SEM. 2-Way repeated measures ANOVA with Bonferroni correction were performed to evaluate time, smoking, and interaction effects (* $P < 0.05$ as compared with Pre).

Figure 4. Plasma 5-NO₂-γ-tocopherol concentrations from non-smokers (n = 10) and smokers (n = 11) prior to and following supplementation with 75 mg/d each of RRR-α-tocopheryl acetate and all rac-α-tocopheryl acetate for 6 consecutive evenings

Plasma 5-NO₂-γ-tocopherol (A) and plasma 5-NO₂-γ-tocopherol expressed as change from Pre (B) prior to (Pre), the morning after receiving supplementation on 6 consecutive evenings (Post 1), and on the mornings of d 6 (Post 6) and d 14 (Post 14) during the post-supplementation period. Data are means $\pm$ SEM. 2-Way repeated measures ANOVA with Bonferroni correction were performed to evaluate time, smoking, and interaction effects (* $P < 0.05$ as compared with Pre in smokers, # $P < 0.05$ as compared with non-smokers).

Figure 5. Correlations between plasma 5-NO₂-γ-tocopherol and γ-tocopherol in non-smokers (n = 10) and smokers (n = 11) who ingested α-tocopherol supplements (75 mg/d each of RRR-α-tocopheryl acetate and all rac-α-tocopheryl acetate for 6 consecutive evenings)

Associations between 5-NO₂-γ-tocopherol and γ-tocopherol, stratified by smoking status, for all plasma samples obtained prior to, and at days 1, 6, and 14 following 6 d α-tocopherol supplementation, were visualized by multiple linear regression controlling for within-subject repeated measures.

Figure 6. Plasma nitrate/nitrite concentrations from non-smokers ($n = 10$) and smokers ($n = 11$) prior to and following supplementation with 75 mg/d each of RRR- α -tocopheryl acetate and all rac- α -tocopheryl acetate for 6 consecutive evenings

Plasma nitrate/nitrite were measured prior to supplementation (Pre), the morning after receiving daily supplementation for 6 consecutive evenings (Post 1), and on the mornings of d 6 (Post 6) and d 14 (Post 14) during the post-supplementation period. Data (means $\pm$ SEM) are expressed as change in plasma nitrate/nitrite relative to Pre in non-smokers and smokers. 2-Way repeated measures ANOVA with Bonferroni correction were performed to evaluate time, smoking, and interaction effects ($\# P < 0.05$ as compared with non-smokers).

Table 1

Participant Characteristics.

	Non-smokers (n = 10)	Smokers (n = 11)	P values
Age (y)	21.3 ± 1.6	20.9 ± 0.9	0.83
BMI (kg/m ²)	22.0 ± 1.0	22.7 ± 1.0	0.62
Cigarettes/d	N/A	12.4 ± 1.5	
Plasma Cotinine (ng/mL)	33 ± 7	2357 ± 596	0.00
Serum Aspartate Aminotransferase (U/L)	21.0 ± 1.4	25.3 ± 5.5	0.48
Serum Lactate Dehydrogenase (U/L)	166 ± 10	197 ± 35	0.42
Serum Alanine Aminotransferase (U/L)	17.2 ± 2.8	19.9 ± 2.5	0.48
Serum Alkaline Phosphatase (U/L)	65.3 ± 7.3	80.1 ± 4.5	0.10
Serum Triglyceride (mg/dL)	94 ± 15	111 ± 17	0.45
Serum Total Cholesterol (mg/dL)	168 ± 5	151 ± 11	0.19
Plasma α-Tocopherol (μmol/L)	19.7 ± 2.0	17.4 ± 2.3	0.46
Plasma γ-Tocopherol (μmol/L)	2.55 ± 0.39	2.99 ± 0.43	0.46
Plasma α-CEHC (nmol/L)	6.25 ± 2.57	6.03 ± 3.23	0.12
Plasma γ-CEHC (nmol/L)	117 ± 19	63 ± 9	0.01
Plasma NGT (nmol/L)	30.6 ± 3.28	61.2 ± 13.73	0.04
Plasma NO _x (μmol/L)	13.1 ± 3.55	23.2 ± 1.23	0.01

Values are means ± SEM. BMI - body mass index, CEHC - carboxyethyl-hydroxychromanol, NGT - 5-NO₂-γ-Tocopherol, NO_x - nitrate/nitrite.