THE ROLE OF OXIDATIVE STRESS AND VITAMIN C ON VITAMIN E UTILIZATION IN HUMANS

DISSERTATION

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the Degree Doctor of Philosophy in the Graduate
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* * * * *

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ABSTRACT

Oxidative and nitrative stress play pivotal roles in the etiology of chronic diseases such as cancer and heart disease. Therefore, investigations to determine the effects of oxidative and nitrative stress on dietary antioxidant utilization are critical for our understanding of chronic disease prevention. To date, the impact of these stressors on vitamin E utilization in humans is controversial. Therefore, for this dissertation, we hypothesized that oxidative and nitrative stress from cigarette smoking will alter vitamin E utilization such that smokers have higher dietary requirements for vitamin E than nonsmokers. Using novel analytical techniques in liquid chromatography/mass spectrometry, we determined that the increased reactive nitrogen species associated with cigarette smoking caused a doubling of plasma nitro-γ-tocopherol. Furthermore, using deuterium labeled α-tocopherols, we observed that smokers, compared with nonsmokers, had a 13% faster plasma α-tocopherol disappearance, suggesting that α-tocopherol functions in vivo as an antioxidant. Further, smokers’ α-tocopherol disappearance rates correlated with plasma ascorbic acid concentrations, suggesting that higher plasma ascorbic acid concentrations could prevent the rapid α-tocopherol disappearance. Therefore, we conducted a double-blind, placebo-controlled, cross-over investigation in smokers and nonsmokers who were provided supplemental ascorbic acid (2-weeks, twice daily, 500 mg) or placebo prior to evaluating vitamin E disappearance kinetics. Plasma ascorbic acid concentrations doubled in both groups in response to the supplement compared to the placebo. In smokers during placebo
treatment, vitamin E disappearance kinetics were again faster than in nonsmokers. However, during vitamin C supplementation, smokers’ vitamin E disappearance was normalized. Plasma F_{2α}-isoprostanes, a marker of lipid peroxidation, remained 34% higher than nonsmokers and vitamin E metabolite production was unchanged in the smokers during supplementation. Taken together, these data suggest that in vivo plasma tocopherols and ascorbic acid interact during lipid peroxidation via the reduction of tocopheroxy radicals to tocopherol by ascorbic acid. Collectively, these investigations have provided evidence that the oxidative stress from cigarette smoking increases vitamin E utilization, justifies higher dietary intakes of vitamins E and C among smokers, and warrants further research to determine if other reductive antioxidants can interact with vitamin E and further modulate vitamin E kinetics.
DEDICATION

To Jenny, my loving wife, for all of her love, support, encouragement, and compassion as I achieved my eventual success
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<td>α</td>
<td>alpha</td>
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<tr>
<td>γ</td>
<td>gamma</td>
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<tr>
<td>HPLC or LC</td>
<td>high pressure liquid chromatography</td>
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<td>LC/MS</td>
<td>high pressure liquid chromatography/mass spectrometry</td>
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<td>NGT</td>
<td>nitro-γ-tocopherol</td>
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<td>RDA</td>
<td>Recommended Dietary Allowance</td>
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<td>RNS</td>
<td>reactive nitrogen species</td>
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<td>ROS</td>
<td>reactive oxygen species</td>
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CHAPTER 1

INTRODUCTION
1.1. OVERVIEW

Oxidative stress, along with or independent of low dietary antioxidant intake, is often an implicating factor in inadequate antioxidant status among humans. Much of the research regarding dietary antioxidants is surrounded by the considerable evidence that antioxidant rich foods, such as fruits and vegetables, might serve a protective role against the development and/or progression of various chronic diseases such as cancer (Block et al., 1992).

Cigarette smoking is a potent exogenous source of oxidative stress experienced by humans. Much research has been conducted in vivo and in vitro to evaluate if cigarette smoking alters dietary antioxidant utilization. However, the results regarding the vitamin E utilization among cigarette smokers compared to nonsmokers have been limited and equivocal. Therefore, well-designed human clinical studies evaluating vitamin E utilization under conditions of oxidative stresses such as cigarette smoking are imperative. Results from such studies will help to determine if a relationship between oxidative stress and vitamin E utilization exists and whether or not cigarette smokers have higher dietary requirements of vitamin E compared to nonsmokers.
1.2. VITAMIN E

**Definition and Overview**

The term vitamin E is used to describe eight lipophilic, naturally occurring compounds which include four tocopherols and four tocotrienols ([Figure 1.1](#)) (Traber and Arai, 1999). Tocopherols have a saturated phytol tail whereas the tocotrienols have an unsaturated tail and within each class, four forms exist as α, β, γ, and δ that differ based on the number and position of methyl groups present on the chromanol head. For example, when \( R_1 = R_2 = R_3 = \text{CH}_3 \) and when the phytol tail is saturated then this homologue of vitamin E is termed \( \alpha \)-tocopherol.

**Brief History of Vitamin E**

Evans and Bishop discovered vitamin E in 1922 as a compound necessary to sustain reproductive ability in rodents (Evans and Bishop, 1922). From their experiment, they determined that rodents fed diets containing rancid fat (e.g. vitamin E deficient) produced offspring that were mostly sterile in the first generation and completely sterile in the second generation. They concluded that fetal resorption occurred despite the presence of normal ovarian structure and function. Additional work (Evans et al., 1936) led to the isolation of \( \alpha \)-tocopherol from wheat germ which had the biologic activity of vitamin E. In the subsequent year, \( \beta \)- and \( \gamma \)-tocopherol were isolated from vegetable oils and it was determined that these vitamin E homologues had lower biological activity than \( \alpha \)-tocopherol (Emerson et al., 1937). While these non-\( \alpha \)-tocopherol forms of vitamin E have been reported to possess
vitamin E biological activity, the purity of these compounds as well as the analytical methods have been recently questioned. Currently, the purity of commercially available γ-tocopherol is identified as ~97% pure with much of the “contamination” attributed to α-tocopherol. Thus, some of the early research regarding vitamin E biological activity needs to be repeated.

Historically, the fetal resorption assay has been used to define vitamin E biological activity despite the fact that the assay is very tedious and time consuming (Machlin, 1991). However, the assay does provide useful information since it determines the vitamin E biologic activity and quantifies the amount of vitamin E necessary to maintain the maximal number of live fetuses. While vitamin E deficiency can be induced in laboratory animals, it is quite difficult to do so in humans. During the 1950’s, Horwitt and others (Horwitt, 1960; Horwitt et al., 1963; Horwitt et al., 1956a) attempted to study the effects of chronically low vitamin E intakes among hospitalized volunteers. After nearly two years of the six-year long investigation, plasma vitamin E decreased into the deficient range, but anemia did not develop despite increased sensitivity of erythrocytes to hydrogen peroxide induced hemolysis.

Despite the efforts of chronic dietary vitamin E restriction in humans, symptoms of vitamin E deficiency such as peripheral neuropathy, spinocerebellar ataxia, skeletal myopathy and pigmented retinopathy have not been observed in the laboratory. In addition, free living humans usually only become vitamin E deficient secondary to other pathologies including fat maldigestion disorders (Sokol et al., 1984), dysfunctional lipid metabolism (Rader and Brewer, 1993) and in those with severe protein-energy malnutrition (Kalra et al., 1998). However, the discovery of the α-tocopherol transfer protein and its rarely occurring mutation has led to the
identification of vitamin E deficiency independently of other pathologies (Cavalier et al., 1998).

**Vitamin E Functions**

The most well known biological function of vitamin E is its ability as a chain-breaking antioxidant that ceases the propagation of lipid peroxidation (Burton and Ingold, 1989) (Figure 1.2). Under this function, vitamin E acts as a peroxyl radical scavenger and is able to protect polyunsaturated fatty acids (PUFAs) from lipid peroxidation (Burton et al., 1983). In fact, Buettner (Buettner, 1993) elegantly described that vitamin E is able to “outcompete” the propagation reactions and that a single vitamin E molecule should be able to protect ~1000 lipid molecules from the chain reaction propagation step. This phenomenon is due to the higher rate constant between vitamin E and peroxyl radicals compared to the rate constant between PUFAs and peroxyl radicals.

Work by Burton et al. indicated that α-tocopherol is the most potent biological form of the tocopherols based on methodology that quantifies the inhibited autooxidation of styrene (e.g. peroxyl radical generation) (Burton et al., 1985). The potencies of the tocopherols were reported in the following order: \( \alpha > \gamma > \beta > \delta \) with respective rate coefficients of 320, 140, 130, and \( 44 \times 10^4 \) (M\(^{-1}\) s\(^{-1}\)). It was concluded from this investigation that α-tocopherol was the superior antioxidant because it contains three methyl groups on the chromanol head that function to stabilize phenoxy radicals unlike the other tocopherols which are lacking one or more methyl groups.

In addition to α-tocopherol’s antioxidant ability, α-tocopherol may have a contributory role in cell signaling. In particular, it appears that protein kinase C, a protein involved in cell proliferation and differentiation, may be inhibited by α-
tocopherol in smooth muscle cells (Boscoboinik et al., 1991; Chatelain et al., 1993), monocytes (Devaraj et al., 1996), and platelets (Freedman et al., 1996). Other proteins that have been reported to be affected by α-tocopherol include VCAM-1 and ICAM-1 (Cominacini et al., 1997) which are two proteins that are important factors in cardiovascular disease risk since they cause the adhesion of certain blood cell components to the vascular endothelium. In addition, some evidence also exists to suggest that vitamin E upregulates the expression of both cyclooxygenase-1 (Chan et al., 1998b) and phospholipase A₂ (Chan et al., 1998a). Lastly, NF-κB, a redox sensitive transcription factor, has been shown to be inhibited by α-tocopherol succinate (Suzuki and Packer, 1993). However, the results of this investigation indicated that this effect was not observed for α-tocopherol or α-tocopheryl acetate. In contrast, it was reported in an animal investigation of Phenobarbital-induced oxidative stress that α-tocopherol acetate in doses of 50 and 250 mg/kg in the diet decreased liver NF-kB activation without affecting other endogenous antioxidant systems (Calfee-Mason et al., 2002).

As for γ-tocopherol, evidence has been reported to suggest that it as well as its physiological metabolite, γ-CEHC (γ-carboxy-ethyl-hydroxy-chroman), may possess anti-inflammatory activity (Jiang and Ames, 2003; Jiang et al., 2000). It was reported (Jiang et al., 2000) that γ-tocopherol and γ-CEHC effectively inhibited cyclooxygenase activity in lipopolysaccharide-stimulated macrophages and interleukin-1β-stimulated epithelial cells. However, it should be noted that the concentrations of γ-CEHC used in these experiments were non-physiological. Additional work (Jiang and Ames, 2003) also demonstrated that γ-tocopherol treatment reduced the synthesis of prostaglandin E₂ and leukotriene B₄ at the site of inflammation in a rodent model of arthritis.
Biological Fates of Vitamin E

*In vivo*, vitamin E has four biological fates which include the unaltered excretion into the bile and feces, metabolism, oxidation, and nitration in the case of γ-tocopherol. The metabolism of vitamin E occurs via a cytochrome P450 dependent pathway that is incompletely understood (Birringer et al., 2001). While the metabolic end products for α-tocopherol (Schultz et al., 1995) and γ-tocopherol (Wechter et al., 1996) have been identified as α- and γ-CEHC respectively, the specific cytochrome P450 isoform utilized to initiate tocopherol metabolism remains subject to debate (Birringer et al., 2001; Sontag and Parker, 2002). Regardless of the isoform utilized, it appears that the tocopherols’ metabolism is initiated by ω-oxidation followed by stepwise β-oxidation until α- or γ-CEHC is formed (Figure 1.3) (Birringer et al., 2001). Hopefully, investigations of tocopherol metabolism will provide evidence for CEHC as a marker for evaluating *in vivo* tocopherol status. Currently, it has been reported that the excretion of α-CEHC is minimal unless individuals are supplemented with α-tocopherol (Radosavac et al., 2002). Furthermore, it appears that for metabolism to be activated, a plasma threshold of 30-40 µM α-tocopherol must be reached (Schuelke et al., 2000). In comparison to α-tocopherol, it appears that γ-tocopherol is more rapidly metabolized. While γ-tocopherol typically represents 10-20% of the total plasma tocopherols (Traber and Kayden, 1989), urinary excretion of γ-CEHC can be observed at levels >10-fold higher than α-CEHC in unsupplemented individuals (Galli et al., 2002). In addition, α-tocopherol supplementation also appears to increase γ-tocopherol metabolism as is noted by higher urinary γ-CEHC excretion and the reduction in plasma γ-tocopherol concentration (Morinobu et al., 2003).

In contrast to metabolism, vitamin E may also undergo oxidation. The hydroxyl group found on the chromanol head of vitamin E provides the antioxidant
capacity but it also serves as a target for reactive species resulting in oxidation of vitamin E. By virtue of free radical scavenging, α-tocopherol undergoes one electron oxidation to form an intermediate tocopherol radical which then may subsequently be oxidized by a second electron to yield various quinone products (Liebler et al., 1996). α-Tocopherol oxidation products are varied, but measurable species include epoxy-α-tocopherolquinones, α-tocopherolquinone, and α-tocopherolhydroquinone. Of these, it appears that α-tocopherolquinone is the most abundant product. It is formed by the hydrolysis of either a tocopherone intermediate or a tocopheroxylium cation which was previously created by reactions with a one- or two-electron oxidant, respectively (Terentis et al., 2002). In the absence of a suitable reducing agent such as ascorbic acid, it appears that α-tocopherolquinone can be conjugated by glutathione-S-transferase which may enable delivery to a site for metabolism or allow for excretion into the bile (Arita et al., 1998).

Little data have accumulated regarding in vivo α-tocopherol oxidation. This is probably due to the fact that these oxidation products can be artifactualy created during the extraction process. Since these oxidation products occur in nominal quantities, even a 1% oxidation of α-tocopherol could significantly increase α-tocopherolquinone levels (Mottier et al., 2002). Despite these difficulties, α-tocopherol oxidation products have been reported from human atherosclerotic lesions (Terentis et al., 2002) and in human plasma after a 1-hour endurance run (Mottier et al., 2002).

In addition to oxidation, nitration of vitamin E may also occur. γ-Tocopherol, unlike α-tocopherol, can trap reactive nitrogen species because it has an unsubstituted position on the chromanol ring (Christen et al., 2002). As a result of the interaction between γ-tocopherol and reactive nitrogen species such as peroxynitrite, 5-nitro-γ-
tocopherol can be formed (Hoglen et al., 1997). Thus, work has been conducted to determine if 5-nitro-\(\gamma\)-tocopherol may serve as a useful biomarker for peroxynitrite interactions and in conditions of inflammation as with the measurement of nitrotyrosine. While more work is warranted in this area, it has been reported that 5-nitro-\(\gamma\)-tocopherol is elevated in zymosan-treated rats (Christen et al., 2002), in the plasma and carotid plaques of patients with coronary artery disease (Morton et al., 2002), and in brains collected post-mortem from patients with Alzheimer’s disease (Williamson et al., 2002).

\textbf{U.S. Dietary Intakes and Recommendations of Vitamin E}

Currently, the dietary recommendations of vitamin E for humans are limited only to \(\alpha\)-tocopherol because the other forms of vitamin E are poorly recognized by the \(\alpha\)-tocopherol transfer protein and they can not be converted to \(\alpha\)-tocopherol (Institute of Medicine (U.S.). Panel on Dietary Antioxidants and Related Compounds., 2000). Based on the available data, the Recommended Dietary Allowance for men and women was established at 15 mg/day of \(\alpha\)-tocopherol. By extrapolation, it was determined that this quantity would achieve plasma \(\alpha\)-tocopherol concentrations that would be sufficient to prevent hydrogen peroxide induced erythrocyte lysis. In an effort to make dietary recommendations as accurate as possible, the stereochemistry of \(\alpha\)-tocopherol was also considered when the formulations for recommendations were developed (Institute of Medicine (U.S.). Panel on Dietary Antioxidants and Related Compounds., 2000). While naturally occurring \(\alpha\)-tocopherol exists only as \textit{RRR-\(\alpha\)-tocopherol}, synthetic preparations result in a \textit{racemic} mixture of eight stereoisomers since the \(\alpha\)-tocopherol phytol tail contains three chiral centers (Figure 1.4). Since 2\(S\)-stereoisomers of \(\alpha\)-tocopherol are not maintained in the plasma or tissues they were
not included in the definition of active components of vitamin E and the dietary recommendations were limited to the 2R-stereoisomers only.

Based on the current dietary recommendations, it appears that Americans are not consuming diets sufficient in α-tocopherol (Institute of Medicine (U.S.). Panel on Dietary Antioxidants and Related Compounds., 2000). Data compiled from the Third National Heath and Nutrition Examination Survey indicated that median vitamin E intakes (as α-tocopherol) from food alone for men and women age 19-30 years were only 9.4 and 6.4 mg, respectively (Institute of Medicine (U.S.). Panel on Dietary Antioxidants and Related Compounds., 2000). However, it is likely that these might be underestimated due to several sources of measurement error which include the underreporting of total energy (Mertz et al., 1991) and fat intake (Briefel et al., 1997), the amounts of fats and oils used in food preparation, the uncertainty of the specific oils consumed, and inaccuracies in the food composition databases (Institute of Medicine (U.S.). Panel on Dietary Antioxidants and Related Compounds., 2000). Collectively, Americans are probably not meeting the dietary recommendations for α-tocopherol intake and the available data regarding dietary intakes of α-tocopherol are probably misleading and inaccurate.

**Intestinal Absorption and Secretion**

Since vitamin E is lipophilic, its absorption from the intestinal lumen is dependent on processes that enable fat digestion and uptake into the enterocytes. In addition to the pancreatic esterases that are required to cleave fatty acids from triglycerides, secretion of bile acids is equally important, as they are necessary for the formation of mixed micelles which make the absorption of vitamin E possible (Traber, 1999). In fact, the absence in either of these components results in poor vitamin E absorption, which is why vitamin E deficiency can be observed in patients with biliary
obstruction, cholestatic liver disease, pancreatitis or cystic fibrosis (Sokol, 1993).

Upon enterocyte uptake of vitamin E, absorption into the lymphatic system is then dependent on chylomicron synthesis and secretion. In the enterocytes, chylomicrons containing triglycerides, cholesterol, phospholipids, and apolipoprotein are synthesized (Cohn et al., 1988). During that process, lipophilic compounds such as vitamin E and carotenoids are incorporated into the chylomicron and then secreted into the lymph. In healthy individuals, the absorption of vitamin E has been estimated to range between 15-45% using radioactive $\alpha$-tocopherol (Blomstrand and Forsgren, 1968). However, in thoracic-duct-cannulated rats, the absorption of vitamin E is less efficient as the amount ingested increases (Traber et al., 1986).

**Hepatic Secretion of Vitamin E**

Differences in plasma concentrations of the various forms of vitamin E were initially thought to be due to differences in intestinal absorption (Traber, 1999). However, investigations using deuterated tocopherols have demonstrated that discrimination is not due to enterocyte uptake or chylomicron secretion (Traber et al., 1992; Traber et al., 1990a). In fact, evidence indicates that the liver is responsible for the preferential secretion of $\alpha$-tocopherol into the plasma (Traber et al., 1990b). Furthermore, it has been reported that VLDL (very low density lipoprotein), containing newly absorbed vitamin E is secreted by the liver into the plasma (Kayden and Traber, 1993).

The likely cause for hepatic discrimination of vitamin E is probably attributed to the $\alpha$-tocopherol transfer protein (Institute of Medicine (U.S.). Panel on Dietary Antioxidants and Related Compounds., 2000). This protein was first identified by Catignani and Bieri (Catignani and Bieri, 1977), purified and characterized from liver cytosol (Sato et al., 1991; Yoshida et al., 1992), and more recently has been
crystallized (Min et al., 2003). The \(\alpha\)-tocopherol transfer protein is expressed mainly in the liver (Arita et al., 1995) and purportedly its function is to preferentially transfer/secrete \(\alpha\)-tocopherol from the liver to the plasma (Traber, 2004) by a mechanism incompletely understood. In comparison to \(\alpha\)-tocopherol, other tocopherols bind with significantly less affinity to this protein, thus providing a suitable explanation for hepatic discrimination of the various vitamin E homologues. In the absence of the \(\alpha\)-tocopherol transfer protein, such as in knock-out mice models (Schock et al., 2004) or humans with a genetic defect for this protein (Cellini et al., 2002), the result is vitamin E deficiency.

**Interaction Of Vitamins E and C**

No convincing evidence exists to support that vitamin E and C directly interact under *in vivo* conditions. It has been reported that guinea pigs fed two concentrations of deuterium-labeled \(\alpha\)-tocopherol and three varying concentrations of ascorbic acid did not show an interaction between these nutrients (Burton et al., 1990b). However, lack of oxidative stress applied to the guinea pigs may have been the limitation of this investigation. Alternatively, *in vitro* investigations conducted under conditions of varying oxidative stress have demonstrated the ability of ascorbic acid to either spare \(\alpha\)-tocopherol from oxidation (Frei et al., 1989a; Huang and May, 2003; May et al., 1998) or to enable \(\alpha\)-tocopherol regeneration from its oxidized form (Bisby and Parker, 1995). From these investigations, it is believed that the \(\alpha\)-tocopheroxyl radical formed within micellar and bilayer membrane systems could be effectively recycled back to \(\alpha\)-tocopherol by ascorbic acid found within the aqueous phase.
1.3. OXIDATIVE STRESS AND CIGARETTE SMOKE

Oxidative stress as it relates to aerobic organisms is commonly defined as a disturbance among antioxidant defenses and the production of free radicals (Halliwell and Gutteridge, 1999). An imbalance in favor of free radicals is often an exacerbating factor for a variety of chronic diseases such as heart disease, cancer and diabetes (Betteridge, 2000). This disturbance may arise from diminished antioxidants, increased production of reactive oxygen species/reactive nitrogen species, or in many cases from a combination of these two factors. While there are numerous sources of oxidative stress, both endogenous and exogenous, cigarette smoking is one of the most potent and well described sources of oxidative stress in humans. Therefore, cigarette smoke is often used as a model to evaluate the negative consequences of oxidative stress on antioxidant defenses.

Recent data indicated that cigarette smoking is the leading cause of death in the United States followed by poor diet and physical inactivity (Mokdad et al., 2004). It was estimated that 435,000 deaths (18.1% of total deaths) were attributed to cigarette smoking. In addition, there has been a relatively small decline in smoking prevalence and more than 22% of the United States’ population (~60 million) continues to partake in this adverse behavior. As a consequence, costs associated with cigarette smoking are estimated to range between $50-73 billion in annual medical-care expenditures making it a huge economic burden for the health-care industry (Richards, 2001).

The adverse health effects of cigarette smoke have been attributed to its enormous oxidative burden on antioxidant defenses. Cigarette smoke contains 4800
identified compounds of which 69 are considered carcinogenic (Hoffmann et al., 2001). From a single puff, cigarette smoke contains $10^{14}$ and $10^{15}$ free radicals in the tar and gas phases, respectively (Church and Pryor, 1985). The prominent radicals in the tar phase consist of a system containing low molecular weight quinones (Q), hydroquinones (QH$_2$), and semiquinones (QH$^*$) (Pryor, 1997). The semiquinone radical is relatively long-lived and has the ability to reduce molecular oxygen to superoxide which may then eventually give rise to hydrogen peroxide and hydroxyl radical. Alternatively, the gas phase cigarette smoke consists of mostly carbon and oxygen centered radicals which appear to be more reactive than tar radicals (Pryor et al., 1983). In addition, the gas phase also contains approximately 500 ppm of nitric oxide which may slowly undergo oxidation to form nitrogen dioxide (Cueto and Pryor, 1994) or react with superoxide to generate peroxynitrite (Beckman et al., 1990).

In short, cigarette smoke contains numerous oxidants that have the capability of interacting with various biomolecules to cause adverse biological effects. Some of the more prominent targets of cigarette smoke include DNA, RNA, lipids, amino acids, proteins, dietary antioxidants and various endogenously synthesized biomolecules such as glutathione and $\alpha_1$-antiprotease (Church and Pryor, 1985; Cross et al., 1999; Cross and Traber, 1997; Pryor et al., 1984; Traber et al., 2000).
1.4. IMPACT OF CIGARETTE SMOKE ON VITAMIN E STATUS

*In vitro* investigations strongly suggest that cigarette smoke depletes α-tocopherol from human plasma (Eiserich et al., 1995; Handelman et al., 1996). For example, plasma exposed to cigarette smoke for 3- or 9-hours resulted in a 20% and 70% decrease in α-tocopherol, respectively (Handelman et al., 1996). Similarly, it has been demonstrated (Eiserich et al., 1995) that α-tocopherol decreased by ~20% after nine puffs of cigarette smoke were administered to plasma. However, in this investigation, the decrease in α-tocopherol was only apparent after ascorbic acid was first depleted which is suggestive that ascorbic acid might serve as the first line of defense against cigarette smoke.

However, despite the strong oxidant nature of cigarette smoke, the relationship between cigarette smoking and vitamin E status in humans remains controversial. Some investigations, among unsupplemented individuals, have indicated lower plasma vitamin E concentrations in smokers (Bolton-Smith et al., 1991; Mezzetti et al., 1995) while other investigations report no differences (Dietrich et al., 2003; Lykkesfeldt et al., 2000; Ross et al., 1995). Additionally, dietary intakes of vitamin E did not appear to differ between smokers and nonsmokers after adjustment for various demographic factors (Wei et al., 2001). Regardless, supplementation for 2-months with α-tocopherol in smokers resulted in elevations of plasma α-tocopherol and a subsequent decrease of urinary lipid peroxidation markers (Huang et al., 2002) which is strongly suggestive of an antioxidant role for α-tocopherol despite a lack of evidence to suggest that smokers utilize α-tocopherols any differently than nonsmokers.
In recent years, investigators have hoped that the use of deuterium-labeled vitamin E will enable them to conduct more precise investigation regarding vitamin E metabolism and utilization in humans since plasma deuterated tocopherols can be monitored independently of dietary tocopherols (Burton et al., 1990a). To date, two clinical studies have attempted to discern if cigarette smoking does in fact cause a more rapid depletion of deuterated α-tocopherol from plasma (Munro et al., 1997; Traber et al., 2001). In the first trial (Munro et al., 1997), supplemented smokers and nonsmokers on a single occasion with deuterated α-tocopherols and then collected blood samples at 6, 12, and 27 h following the supplement. While smokers had lower plasma deuterated α-tocopherol concentrations at each of the time points studied, it could not be elucidated whether these differences were due to differences in α-tocopherol absorption or due to its faster plasma clearance. In order to further clarify these differences, smokers and nonsmokers were supplemented with deuterated α-tocopherols for seven days and collected fasting blood samples on select days for three weeks in an effort to evaluate differences in fractional disappearance rates (Traber et al., 2001). While both groups achieved similar plasma deuterated α-tocopherol concentrations as a result of supplementation and the smokers had a faster rate of deuterated α-tocopherol disappearance, this latter finding was not statistically significant because the investigation was likely underpowered.
1.5. CONCLUSION

While it is obvious that cigarette smoke is a strong oxidative stress, its effects on vitamin E utilization under in vivo situations are not as clear. Cigarette smoking can result in the formation of lipid hydroperoxides as well as other markers of oxidative stress and supplementation with vitamin E appears to have the ability to ameliorate these effects. Therefore, if vitamin E is truly functioning as an antioxidant, then cigarette smokers should have faster plasma vitamin E rates of disappearance, lower plasma vitamin E concentrations, and perhaps lower urinary α-CEHC excretion. However, this is often not observed, perhaps due to methodological limitations. Therefore, additional clinical investigations are warranted to properly investigate the impact of oxidative stress on vitamin E status and determine if such a relationship exists.
Figure 1.1. Generalized structure of tocopherols and tocotrienols. Structures are similar except tocopherols have a saturated phytyl tail and tocotrienols have an unsaturated phytyl tail.
Figure 1.2. Chain-breaking antioxidant ability of α-tocopherol.
The ability of α-tocopherol to terminate peroxyl radical regeneration is among its most well known biological functions. Adapted from Burton and Traber (Burton and Traber, 1990). Abbreviations: R’, carbon-centered radical; ROO’, peroxyl radical; R-OO-H, lipid hydroperoxide; RH, polyunsaturated fatty acid; α-TOH, reduced form of α-tocopherol; α-T’, oxidized form of α-tocopherol.
Figure 1.3. Metabolic pathway of α-tocopherol
After ω-oxidation, α-tocopherol metabolism continues through five cycles of β-oxidation until the final urinary product, α-CEHC, is formed (Birringer et al., 2001). Abbreviations: α-CMHHC (α-carboxy-methyl-hexyl-hydroxy-chroman), α-CMBHC (α-carboxy-methyl-butyl-hydroxy-chroman), and α-CEHC (α-carboxy-ethyl-hydroxy-chroman).
Figure 1.4. \( \alpha \)-Tocopherol stereochemistry.
Shown in the figure is \( RRR \)-\( \alpha \)-tocopherol. Due to the three chiral centers present along the phytyl tail, eight possible stereoisomers may arise: \( RRR-, RSR-, RRS-, RSS-, SRR-, SSR-, SRS-, \) and \( SSS-\alpha \)-tocopherol.
CHAPTER 2

5-NITRO-γ-TOCOPHEROL INCREASES IN HUMAN PLASMA EXPOSED TO CIGARETTE SMOKE IN-VITRO AND IN-VIVO
2.1. SUMMARY

We hypothesized that the high concentrations of reactive nitrogen species in cigarette smoke and the known stimulatory effects of cigarette smoke on the inflammatory immune systems would lead to the formation of 5-nitro-γ-tocopherol (NGT). In order to assess γ-tocopherol nitration, human plasma was exposed in vitro to gas phase cigarette smoke (GPCS) or air up to 6 h. A liquid chromatography-mass spectrometry (LC/MS) method was developed to quantitate NGT. Detector response was linear from 0.1 to 3 pmol NGT, with a detection limit of 20 fmol. Following a 1 h lagtime, 6 h plasma exposure to GPCS depleted ~75% of α-T, ~60% of γ-T and increased NGT from 4 to 227 nmol/L. The increase in NGT accounted for ~20% of the γ-T decrease. NGT also correlated (R² = 0.9043) with nitrate concentrations in GPCS-exposed plasma. The physiologic relevance of NGT was evaluated in a group of healthy humans. Smokers (n=15) had plasma NGT concentrations double those of non-smokers (n=19), regardless of corrections using lipids or γ-T; plasma α-T and γ-T concentrations were similar between the groups. Our results show that LC/MS can be successfully used for NGT quantitation in biologic samples. Importantly, NGT in smokers’ plasma suggests that cigarette smoking causes increased nitrosative stress.
2.1. INTRODUCTION

Cigarette smoking is a well known health hazard that is directly related to the pathogenesis of various chronic diseases (Mendez-Alvarez et al., 1998). Yet, nearly 50 million individuals in the United States partake in this behavior (Karnath, 2002). The harmful effects of smoking have been attributed to the abundance of reactive oxygen species (ROS) and reactive nitrogen species (RNS) that readily react with various biomolecules. In a single puff, cigarette smoke contains $10^{14-15}$ reactive oxygen species, ~500 ppm nitric oxide (NO) and other reactive nitrogen oxides (Pryor, 1997). In addition, cigarette smoke causes an inflammatory response which further perpetuates radical production (Alberg, 2002; Brown et al., 1997). NO may also react with superoxide to form peroxynitrite (Beckman et al., 1990) which consequently can then react with various biomolecules (Goss et al., 1999).

Vitamin E is a lipophilic, chain breaking antioxidant found within biological membranes that can prevent biological damage (Traber and Packer, 1995). It exists as a mixture of α-, β-, γ-, and δ- tocopherols and tocotrienols that differ in the saturation of the phytanyl tail as well as the number and position of methyl groups found on the chromanol ring (Brigelius-Flohe and Traber, 1999). Of most biological interest are α- and γ-tocopherol since these are the forms that are present in measurable amounts in plasma and tissues (Traber, 1999). In comparison to α-T, γ-T is the major tocopherol found in the diet but is generally found in much lower quantities \textit{in vivo} (Traber and Arai, 1999), typically accounting for 10-20% of the total tocopherol plasma pool (Traber and Kayden, 1989). However, γ-T, unlike α-T, has an unsubstituted position
on the chromanol ring (Figure 1). While this provides \( \alpha \)-T with greater antioxidant potential and bioactivity (Christen et al., 2002), \( \gamma \)-T has a greater ability to trap certain reactive nitrogen oxides (Christen et al., 1997).

It has been reported (Cooney et al., 1993) that \( \gamma \)-T is more effective at detoxification of \( \text{NO}_2 \) than \( \alpha \)-T. Furthermore, subsequent work has demonstrated that 5-nitro-\( \gamma \)-tocopherol (2,7,8-trimethyl-2-(4,8,12-trimethyldecyl)-5-nitro-6-chromanol; NGT) is the major reactive product between peroxynitrite and \( \gamma \)-T (Hoglen et al., 1997). Thus, NGT, like 3-nitro-tyrosine, may serve as a useful \textit{in vivo} biomarker for peroxynitrite interactions. Elevations in NGT have been reported in the plasma of zymosan treated rats (Christen et al., 2002), plasma from patients with coronary artery disease (Morton et al., 2002), and in the brains collected post-mortem from patients with Alzheimer’s disease (Williamson et al., 2002).

\( \gamma \)-T, as well as its metabolite (\( \gamma \)-CEHC; \( \gamma \)-carboxyethylhydroxychroman), possess anti-inflammatory properties because stimulated macrophages and epithelial cells treated with \( \gamma \)-tocopherol were shown to have reduced cyclooxygenase-2 activity as well as reduced prostaglandin \( \text{E}_2 \) (PGE\(_2\)) synthesis (Jiang et al., 2000). Moreover, in rats fed a high \( \gamma \)-T diet (33 mg/kg chow) and subjected to carrageenan-induced inflammation, PGE\(_2\) and leukotriene B\(_4\) synthesis were decreased by 46% and 70%, respectively (Jiang and Ames, 2003).

We hypothesized that the high concentrations of reactive nitrogen species in cigarette smoke and the known stimulatory effects of cigarette smoke on the inflammatory immune systems would lead to the formation of NGT in human plasma, both \textit{in vitro} and \textit{in vivo}. To test this hypothesis, we developed a liquid chromatography/mass spectrometry method to quantitate NGT in plasma exposed to gas phase cigarette smoke \textit{in vitro}, and in plasma from smokers.
2.3. MATERIALS AND METHODS

Subjects

This study was approved by the Institutional Review Board at Oregon State University. All participants provided informed consent prior to enrollment in the study. Thirty four volunteers (13 females, 21 males) were recruited from advertisements placed in a local newspaper. Smokers (5 females, 10 males) were selected based on having smoked greater than 10 cigarettes per day. Nonsmokers (8 females, 11 males) were selected based on that they had never been a smoker and were not currently residing with a smoker. Subjects had no history of taking any dietary supplements within the last year and the physical characteristics of these subjects are presented in Table 2.1. Routine serum blood chemistry assays were performed on all of the subjects at Good Samaritan Hospital (Corvallis, OR) (Table 2.2). All serum chemistries were within normal limits with the exception of two subjects having moderately elevated total cholesterol.

Study Design and Procedures

In vitro cigarette smoke exposed plasma collection

Blood (140 mL) was drawn from the antecubital arm vein into tubes containing 0.05 mL 15% K₃ EDTA (Vacutainer, Becton Dickinson, Franklin Lakes, NJ), from 1 normolipidaemic female subject by a trained phlebotomist. The blood was centrifuged at 500 x g for 15 min at 4°C, at which time the plasma was removed and pooled. For
this experiment, 20 mL aliquots of plasma were placed into two 250 mL Erlenmeyer filter flasks and exposed to either gas phase (filtered) cigarette smoke (GPCS) or air for up to 6 h. The sidearm flask was connected via a Y to a vacuum and to a cigarette, with a filter. Cigarettes used were University of Kentucky (UK) 2R1 research cigarettes that contained 23 mg of tar and 2.2 mg of nicotine per cigarette according to the Federal Tobacco Council. The flask was evacuated to 0.2 kPa, and the vacuum line was then clamped, and the line to the cigarette was unclamped as the cigarette was lit. Cigarette smoke was introduced to the flask for 5 sec, burning ~50% of the cigarette. The flask was clamped and incubated at 37°C for 30 min. Following the incubation time, a 1 ml aliquot was removed and snap frozen in liquid nitrogen. The process was repeated every 30 min up to 150 min, and at 240 and 360 min for a total of 7 sample collections for GPCS. For the sham room air exposed plasma experiment, only four samples were collected (0, 60, 180, and 360 min).

In vivo cigarette smoke exposed plasma collection

All subjects provided a blood sample after fasting overnight. Smokers were instructed to abstain from smoking for one hour prior to sample collection. Blood was drawn from the antecubital arm vein of 34 subjects (as described above) by a trained phlebotomist into tubes containing 0.05 mL 15% K3EDTA (Vacutainer, Becton Dickinson, Franklin Lakes, NJ). Plasma was promptly separated by centrifugation at 4°C for 15 m at 500 x g, snap frozen in liquid nitrogen, and stored at -80°C until analyzed.

Materials

HPLC-grade solvents were obtained from Fisher (Fair Lawn, NJ). Ascorbic acid, potassium hydroxide (KOH), and butylated hydroxy toluene (BHT) were
purchased from Sigma-Aldrich (St. Louis, MO). α-Tocopheryl acetate and γ-tocopherol standards were gifts provided by Dr. James Clark of Cognis Nutrition and Health, LaGrange, IL. NGT was synthesized by the methods previously described (Christen et al., 1997). The material produced by this method was confirmed by EI-MS, ¹H- and ¹³C-NMR. The purity of NGT at the time of preparation and analysis was > 98%. all rac-α-5,7,8-(CD₃)₃ tocopheryl acetate was provided by Dr. Carolyn Good of General Mills and was synthesized by Isotec Inc. (Miamisburg, OH). The isotopic purity was found to be 88.4% d₉ and the remainder d₈.

**Quantitative analysis of tocopherols**

Plasma vitamin E was extracted according to a previously described method (Podda et al., 1996b). In brief, plasma was saponified with alcoholic KOH, and the tocopherols were extracted with hexane. After centrifugation at 500 x g for 5 min at 4°C, the hexane layer was evaporated under N₂ gas, and the residue resuspended in 1:1 MeOH:EtOH. The tocopherol contents were analyzed by LC/MS according to the procedures outlined below.

For the *in vitro* plasma experiment, 100 µL plasma was used for tocopherol determination, with 30 µL injected out of the 100 µl resuspension. For the *in vivo* plasma experiment, 1 mL plasma was used. After the samples were taken to dryness under nitrogen gas, the pellet was redissolved in 100 µL 1:1 MeOH:EtOH and 50 µL injected for NGT determination. For α- and γ-tocopherol analyses, because of their substantially higher extract concentrations, 10 µL of the resuspension was aliquoted, diluted 20 fold, and 5 µL injected.

Calibration curves were prepared using the authentic standards and *all rac*-α-5,7,8-(CD₃)₃ tocopherol was used as the internal standard. Concentrations of the tocopherol standards were determined spectrophotometrically (Beckman DU Series
600, Fullerton, CA) using: \( \varepsilon_{292 \text{ nm} \text{ EtOH}} = 3,270 \text{ M}^{-1} \text{ cm}^{-1} \) for \( \alpha \)-tocopherol, \( \varepsilon_{298 \text{ nm} \text{ EtOH}} = 3,810 \text{ M}^{-1} \text{ cm}^{-1} \) for \( \gamma \)-tocopherol, and \( \varepsilon_{410 \text{ nm} \text{ EtOH}} = 1,976 \text{ M}^{-1} \text{ cm}^{-1} \) nm for NGT (Christen et al., 2002; Cooney et al., 1995b; Podda et al., 1996b).

**Liquid Chromatography**

For chromatographic separation, a Waters 2690 Separations Module (Milford, MA) was used. The unit contained a cooled autosampler with a 100 \( \mu \text{L} \) sample loop and a degassing unit. Instrument control and acquisition were performed using Waters Masslynx software (version 3.4). Tocopherols were separated using a Symmetry® LC-18 column (Waters, Millford, MA) 4.6 x 75 mm, 3.5 \( \mu \text{m} \) with a Symmetry® Sentry™ guard column 3.9 x 20 mm, 5 \( \mu \text{m} \). An isocratic mobile phase delivery system was used consisting of 100% methanol delivered at a flow rate of 1 ml/min, with a total run time of 15 minutes.

**Mass-spectrometry**

For mass spectral analysis, a ZQ 2000 single-quadrupole mass spectrometer (Micromass, Manchester, England) was used, with the Micromass MassLynx NT version 3.4 software, using a modification of the described method (Lauridsen et al., 2001a). The source was equipped with an atmospheric pressure chemical ionization (APCI) probe, set to negative ionization mode. The analysis parameters were set as follows: corona discharge electrode 15.0 \( \mu \text{A} \), APCI probe temperature 450° C and heater gas (nitrogen) 350 L/hr, nebulizer gas (nitrogen) 80 psi, cone gas (nitrogen) 25 L/hr and voltage -35 V; with a dwell time of 0.20 s per compound. All samples were analyzed using single ion recording. Mass-to-charge \( (m/z) \) ratios were obtained as follows: \( d_0-\alpha \)-tocopherol, \( m/z \) 429.4, \( d_0-\alpha \)-tocopherol, \( m/z \) 438.4, \( d_0-\gamma \)-tocopherol,
m/z 415.4, 5-nitro-γ-tocopherol, m/z 460.4, and 5-nitro-γ-tocopherol fragment, m/z 194.4.

Quantitative analysis of lipids

Plasma free F2-isoprostanes were measured using the Cayman 8-isoprostane EIA kit (Cayman Chemical, Ann Arbor, MI, USA) and performed in accordance with manufacturer’s specifications. Samples and standards were read from an ELISA microplate with a Spectramax 190 microplate reader (Molecular Devices; Sunnyvale, CA) at 405 nm. Data were corrected for losses using a radioactive internal standard and calculations made according to kit instructions. Plasma triglycerides and total cholesterol were determined by standard clinical assays (Sigma, St. Lois, MO).

Total Nitrate/Nitrite Determination

Total nitrite/nitrate (NO₂⁻/NO₃⁻) was measured as an index of NO status (NOₓ). This was accomplished using a colorimetric kit purchased from Cayman Chemicals (Ann Arbor, MI) and measured in accordance with manufacturer’s instructions.

Statistical Analysis

Statistical analysis was performed using Minitab (Version 13; State College, PA). A non-paired student’s t-test was used for all of the data analysis. All data are expressed as mean ± SD unless otherwise specified.
2.4. RESULTS

Mass spectrometry detection and method validation

Due to the similarity in structure to unmodified tocopherols, NGT was extracted from plasma as previously described (Podda et al., 1996b). Recovery of NGT added to plasma was 93 ± 16%. In Figure 2.2, a typical chromatogram is shown of authentic tocopherols and the total ion chromatogram (TIC) produced in a single run through the mass spectrometer. For NGT, the detection response was linear from 0.1 to 3.0 pmol injected, with a detection limit of 20 fmol injected. Simultaneously injected α- and γ-Ts were linear from 20 to 260 pmol, and 3 to 36 pmol injected, respectively (Figure 2.3). Compared to positive ionization, negative ionization produced greater sensitivity due to a decrease in background.

Collision induced dissociation (CID), which occurs in the collision cell of a triple quadrupole mass spectrometer, can be simulated using a single quadrupole instrument by increasing the cone voltage and colliding parent compounds with nitrogen gas in the source, causing fragmentation to the corresponding daughter ions. This technique can be used to verify the identification of injected compounds. Injecting the same sample 10 times and increasing the cone voltage prior to each run, we found the increase in the respective daughter ion (m/z 194.4) increased linearly (R² = 0.9991) with the decrease in the corresponding parent ion (m/z 460.4) with the same retention time (Figure 2.4), thereby identifying the compound as NGT.
**In vitro experiment**

To test whether NGT was detectable when extracted from a biological fluid, human plasma was exposed to filtered GPCS or air (sham control). Smoke-exposed human plasma has been previously reported to show decreases in antioxidants, coupled with an increase in lipid peroxidation products (Frei et al., 1991). In addition, cigarette smoke is laden with reactive nitrogen species which is why we chose this model to validate our method for NGT determination. Following a 1 hour lag-time, plasma exposed to GPCS for 6 hours depleted ~75% of the α-T, ~60% of the γ-T and increased NGT from 0.004 to 0.227 µM (**Figure 2.5**). Of the γ-T decrease, the increase in NGT accounted for ~20% of the initial γ-T content. Similarly to what was reported previously (Frei et al., 1991), cigarette smoke exposure depleted plasma ascorbic acid within 30 min (data not shown). After 2 hours, α- and γ-T began to disappear very rapidly, corresponding to a rapid increase in the production of the F₂-isoprostanes (**Figure 2.5**), reflecting an increase in the production of lipid peroxidation products.

Cigarette smoke is known to contain high amounts of reactive nitrogen oxide species (Deliconstantinos et al., 1994), which could potentially react with γ-T, or interact with reactive oxygen species producing potential nitrating species. To better understand this relationship, we measured changes in NOₓ levels in our samples. In the smoke exposed plasma, NGT correlated very highly with the NOₓ levels ($R^2 = 0.9043$). No significant correlations were found in plasma exposed to air.

**In vivo experiment**

NGT, α-T, and γ-T were measured in plasma collected from smokers and non-smokers. Since smoking is known to increase inflammatory markers (Blake and Ridker, 2002; Jialal and Devaraj, 2001) we hypothesized that smokers would have
elevations in NGT. It was determined that NGT was approximately two-fold higher in smokers regardless of whether the data were corrected for total lipids or γ-T (Table 2.3). Furthermore, positive correlations were found between number of cigarettes smoked per day and NGT ($r = 0.415; p = 0.015$), NGT/lipids ($r = 0.370; p = 0.31$), and NGT/γ-T ($r = 0.431; p = 0.011$). No statistical differences were found for either γ-T ($p = 0.822$), or α-T ($p = 0.923$) between smokers and nonsmokers.

Since the nitrating compounds are most likely generated from reactive nitrogen species, once again we measured NOx as an index of NO status. The smokers’ plasma contained significantly higher NOx concentrations compared to non-smokers ($p = 0.043$), however, no correlations were found between smoker plasma NGT and NOx.
2.5. DISCUSSION

Our newly developed LC/MS analysis demonstrated that apparently healthy cigarette smokers had statistically higher NGT regardless of corrections against lipids or \( \gamma \)-T concentration. These findings were noted despite the apparent lack of differences in either \( \alpha \)-T or \( \gamma \)-T among the groups. We chose to study cigarette smokers because they typically have greater activation of inflammatory immune processes and because they are subjected to cigarette smoke, which presents a concentrated source of RNS to respiratory tract tissues. Smokers had statistically higher NO\(_x\) concentrations than nonsmokers, suggesting that smokers are subject to greater nitrosative stress. Interestingly, NGT in nonsmokers and smokers represented only 0.78\% and 1.4\% of \( \gamma \)-T, respectively, which is considerably lower than what has been reported in the plasma of zymosan-treated rats (Christen et al., 2002), suggesting a more intense activation of inflammatory-immune processes by zymosan and/or a more rapid removal of NGT from plasma by humans. However, the fact that NGT is elevated in smokers may provide an alternative marker for nitrosative stress that should be considered in future experiments.

We demonstrate here that NGT can be successfully extracted and measured using LC/MS from samples whether NGT was produced \textit{in vitro} or \textit{in vivo}. HPLC separation of the \( \alpha \)-T, \( \gamma \)-T, and NGT peaks was excellent. Furthermore, since we used an external standard of NGT and an internal standard of \( \text{d}_9 \)-\( \alpha \)-T we were able to sensitively quantify NGT as low of 20 fmol. In addition, all tocopherol measurements had a CV of \(<\ 10\%\).
The results of the *in vitro* experiment support previous work demonstrating that cigarette smoke is a potent source of ROS and RNS that can deplete biological fluid of antioxidants (Eiserich et al., 1995; Frei et al., 1991; Handelman et al., 1996). Human plasma directly exposed to cigarette smoke resulted in the depletion of dietary antioxidants with simultaneous increases in the concentration of isoprostanes and NGT. After 6 hours of smoke exposure, there were 75% and 60% reductions in α-T and γ-T, respectively. In addition, there was more than a 55-fold increase in NGT from baseline. Furthermore, the NGT concentration after 6 hours of smoke exposure accounted for ~20% of the baseline γ-T concentration, suggesting that γ-T is readily nitrated by cigarette smoke. Since nitric oxide readily associates and concentrates in lipophilic membranes (Denicola et al., 2002; Liu et al., 1998) as does γ-T, there may be a membrane protective effect of γ-T against nitrating species.

Interestingly, although ascorbic acid disappeared completely during the first 30 minutes of smoke exposure (data not shown), it took nearly two hours before the depletion of α- and γ-T occurred along with a concomitant increase in the formation of isoprostanes and NGT. This is most likely due to the presence of other water-soluble plasma antioxidants such as uric acid, thiols, and proteins. Given the delay in the formation of these oxidized products (NGT and isoprostanes), it is suggestive that antioxidant defenses must be overwhelmed by ROS and RNS prior to the formation of these oxidized products.

It is quite feasible that γ-T, albeit in low blood concentrations, “traps” harmful nitrogen oxide species and therefore protects other target molecules such as tyrosine from nitration (Christen et al., 1997), especially since it has been reported (Goss et al., 1999) that nitration of γ-T occurs approximately 15-times more readily than the nitration of tyrosine. Therefore, the formation of NGT can be viewed as a positive
biological response in which $\gamma$-T spares other lipophilic biomolecules from being attacked by reactive nitrogen species.

More work is warranted to elucidate the mechanism(s) involved in NGT formation. It is reasonable to believe that NGT might serve as an important lipophilic biomarker for humans since NO is quite lipophilic and peroxynitrite often targets lipophilic biomolecules (Hensley et al., 2000). It should also be considered that $\alpha$- and $\gamma$-T act synergistically as both $\alpha$- and $\gamma$-T function as a powerful lipophilic antioxidants and can react with peroxynitrite (Goss et al., 1999; Hensley et al., 2000); additionally, $\gamma$-T acts an “anti-nitrative” compound, forming NGT. Certainly, NGT is not an exclusive “footprint” of peroxynitrite formation. Although peroxynitrite could be the precursor of nitrogen dioxide, other sources in the chronic inflammatory condition caused by cigarette smoking could include myeloperoxidase (Eiserich et al., 1998), and even smoke itself (Pryor, 1997).
2.6. ACKNOWLEDGMENTS

The subjects receive our thanks for their cooperation. Financial support was received from the California Tobacco Related Disease Research Program (7RT-0160) and NIH DK59576 to MGT.
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Nonsmokers (n = 19)</th>
<th>Smokers (n= 15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wt (kg)</td>
<td>70.9 ± 18.2</td>
<td>72.5 ± 25.7</td>
</tr>
<tr>
<td>Ht (m)</td>
<td>1.7 ± 0.1</td>
<td>1.7 ± 0.4</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>24.7 ± 4.9</td>
<td>23.5 ± 7.1</td>
</tr>
<tr>
<td>Age (y)</td>
<td>20.8 ± 3.7</td>
<td>21.9 ± 7.2</td>
</tr>
<tr>
<td>Cigarettes/day</td>
<td>-</td>
<td>12.8 ± 4.8</td>
</tr>
<tr>
<td>Cholesterol (mmol/L)</td>
<td>4.47 ± 0.78</td>
<td>4.16 ± 1.40</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>1.47 ± 0.78</td>
<td>1.59 ± 1.30</td>
</tr>
</tbody>
</table>

**Table 2.1. Subjects' characteristics**
Data are shown as mean ± SD. Baseline characteristics of smokers and nonsmokers were similar except for cigarettes/day.
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Nonsmokers</th>
<th>Smokers</th>
<th>Normal range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium (mEq/L)</td>
<td>142 ± 2</td>
<td>142 ± 1</td>
<td>135 - 145</td>
</tr>
<tr>
<td>Potassium (mEq/L)</td>
<td>4.2 ± 0.3</td>
<td>4.5 ± 0.5</td>
<td>3.5 - 5.1</td>
</tr>
<tr>
<td>Chloride (mEq/L)</td>
<td>103 ± 1</td>
<td>103 ± 1</td>
<td>100 - 111</td>
</tr>
<tr>
<td>Bicarb (mEq/L)</td>
<td>25.6 ± 1.7</td>
<td>25.8 ± 2.3</td>
<td>22 - 30</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>82 ± 6</td>
<td>81 ± 10</td>
<td>70 - 105</td>
</tr>
<tr>
<td>BUN (mg/dL)</td>
<td>13 ± 3</td>
<td>13 ± 3</td>
<td>6 - 19</td>
</tr>
<tr>
<td>Creatinine (mg/dL)</td>
<td>0.8 ± 0.1</td>
<td>0.8 ± 0.1</td>
<td>0.4 - 1.1</td>
</tr>
<tr>
<td>BUN/creatinine ratio</td>
<td>16 ± 4</td>
<td>17 ± 4</td>
<td>6 - 30</td>
</tr>
<tr>
<td>Uric acid (mg/dL)</td>
<td>5.1 ± 1.4</td>
<td>5.4 ± 1.1</td>
<td>2.4 - 5.7</td>
</tr>
<tr>
<td>Calcium (mg/dL)</td>
<td>9.8 ± 0.5</td>
<td>10.0 ± 0.4</td>
<td>8.4 - 10.2</td>
</tr>
<tr>
<td>Phosphorus (mg/dL)</td>
<td>3.9 ± 0.6</td>
<td>4.1 ± 0.7</td>
<td>2.7 - 4.5</td>
</tr>
<tr>
<td>Total protein (g/dL)</td>
<td>7.4 ± 0.2</td>
<td>7.4 ± 0.4</td>
<td>6.4 - 8.3</td>
</tr>
<tr>
<td>Albumin (g/dL)</td>
<td>4.3 ± 0.4</td>
<td>4.5 ± 0.3</td>
<td>3.4 - 5.0</td>
</tr>
<tr>
<td>SGOT (U/L)</td>
<td>22 ± 6</td>
<td>25 ± 16</td>
<td>0 - 31</td>
</tr>
<tr>
<td>LDH (U/L)</td>
<td>170 ± 32</td>
<td>194 ± 100</td>
<td>94 - 250</td>
</tr>
<tr>
<td>SGPT (U/L)</td>
<td>19 ± 9</td>
<td>25 ± 19</td>
<td>0 - 31</td>
</tr>
<tr>
<td>Alkaline phosphatase (U/L)</td>
<td>76 ± 25</td>
<td>78 ± 14</td>
<td>39 - 117</td>
</tr>
<tr>
<td>Gamma GT (U/L)</td>
<td>16 ± 7</td>
<td>21 ± 16</td>
<td>7 - 33</td>
</tr>
<tr>
<td>Total bilirubin (mg/dL)</td>
<td>0.7 ± 0.4</td>
<td>0.6 ± 0.3</td>
<td>0.0 - 1.0</td>
</tr>
<tr>
<td>Globulin (g/dL)</td>
<td>3.1 ± 0.4</td>
<td>2.9 ± 0.3</td>
<td>2.3 - 3.5</td>
</tr>
</tbody>
</table>

**Table 2.2. Subjects' blood chemistry and hematological values at screening**
Participants had values (mean ± SD) within normal limits.
<table>
<thead>
<tr>
<th></th>
<th>Nonsmokers (n = 19)</th>
<th>Smokers (n = 15)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha$-T (µM)</td>
<td>16.0 ± 4.0</td>
<td>15.9 ± 5.0</td>
<td>0.923</td>
</tr>
<tr>
<td>$\gamma$-T (µM)</td>
<td>1.76 ± 0.98</td>
<td>1.70 ± 0.69</td>
<td>0.822</td>
</tr>
<tr>
<td>$\gamma$-T/lipids (µmol/mmol)</td>
<td>0.29 ± 0.13</td>
<td>0.31 ± 0.16</td>
<td>0.327</td>
</tr>
<tr>
<td>$\alpha$-T/lipids (µmol/mmol)</td>
<td>2.70 ± 0.48</td>
<td>2.79 ± 0.88</td>
<td>0.354</td>
</tr>
<tr>
<td>NGT (nM)</td>
<td>4.03 ± 3.10 (3.90)</td>
<td>8.02 ± 8.33 (6.10)</td>
<td>0.031</td>
</tr>
<tr>
<td>NGT/$\gamma$-T (nmol/µmol)</td>
<td>2.28 ± 1.64 (2.43)</td>
<td>4.25 ± 4.12 (3.31)</td>
<td>0.033</td>
</tr>
<tr>
<td>NGT/lipids (nmol/mmol)</td>
<td>0.66 ± 0.53 (0.657)</td>
<td>1.23 ± 1.04 (1.067)</td>
<td>0.023</td>
</tr>
</tbody>
</table>

Table 2.3. *In vivo* plasma tocopherol concentrations
Median values for NGT are shown in parenthesis.
Figure 2.1. Structures of α-, γ-, and 5-nitro-γ-tocopherols

γ-tocopherol (γ-T) : $R_1 = H$
α-tocopherol (α-T): $R_1 = \text{CH}_3$
5-nitro-γ-tocopherol (NGT): $R_1 = \text{NO}_2$
Single ion recordings were carried out to detect NGT ($m/z$ 460.4), α-T ($m/z$ 429.4) and γ-T ($m/z$ 415.4). The 20 µL injection mixture contained 1.4 pmol NGT, 60.0 pmol α-T, and 6.0 pmol γ-T.
Figure 2.3. Calibration curves for $\alpha$-, $\gamma$-, and 5-nitro-$\gamma$-tocopherol.
Calibration curves were generated simultaneously by mixing authentic NGT, $\alpha$-T, and $\gamma$-T in amounts expected in human plasma samples, such that the molar ratios of the three molecules were approximately 1:250:35, respectively. Single ion recordings were measured by LC/MS. For clarity, insets show the relationship between pmol injected and area counts for NGT and $\gamma$-T, respectively.
Figure 2.4. Cone voltage experiment to identify NGT
The optimum cone voltage for NGT was found to be 40 V. A linear relationship was found ($R^2 = 0.9991$) when correlating the decrease in the parent ion (460.4) to the increase in the corresponding fragment ion (194.4) using the data between the cone voltages of 40 to 60. These data confirm that the peak shown in figure 2.1 contains NGT.
Figure 2.5. In vitro exposure of human plasma to cigarette smoke.

Human plasma was exposed in-vitro to air (♦) or gas phase cigarette smoke (■; GPCS) up to 6 h at 37 °C. Aliquots were taken at intervals and the concentrations of α-tocopherol (α-T, panel A), γ-tocopherols (γ-T, panel B), F2-isoprostanes (F2-IsoP, panel C) and nitrated γ-tocopherol (NGT, panel D) are shown.

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CHAPTER 3

FASTER ALPHA-TOCOPHEROL DISAPPEARANCE KINETICS ARE RELATED TO LOW ASCORBIC ACID STATUS IN CIGARETTE SMOKERS
3.1. SUMMARY

**Background:** Cigarette smokers have enhanced oxidative stress from exposure to cigarette smoke as well as from their increased inflammatory responses.

**Objective:** The objective of this study was to determine if cigarette smoking increases plasma α-tocopherol (α-T) disappearance in otherwise healthy humans.

**Design:** Smokers and nonsmokers (n = 10/group) were supplemented with deuterium labeled α-TAcs (75 mg each d₃-RRR- and d₆-all rac-α-tocopheryl acetates) for six evenings (days -6 to -1). Plasma α-Ts, ascorbic acid, uric acid, and F₂α-isoprostanes were measured from blood samples collected on days -5 through 17. The urinary α-T metabolite, α-CEHC (α-carboxy-ethyl-hydroxy-chroman), was measured on days -6, 0, and 17 from 24 h urine collections.

**Results:** F₂α-isoprostanes were on average ~42% higher in smokers than nonsmokers. At day 0, both groups had similar plasma labeled and unlabeled α-T concentrations. Cigarette smoking resulted in faster plasma α-T disappearance (0.215 ± 0.001 vs 0.191 ± 0.001 pools/day; p < 0.05). α-T disappearance rates correlated with plasma ascorbic acid concentrations in smokers (p = 0.021; R² = 0.509) but not in nonsmokers (p = 0.505; R² = 0.057) despite similar average plasma ascorbic acid concentrations in both groups. By day 17, cigarette smoking resulted in both lower plasma α-T concentrations and a lower urinary excretion of labeled and unlabeled α-CEHCs (p < 0.05).

**Conclusions:** Cigarette smoking caused increased α-T utilization. Greater rates of α-T disappearance in the smokers appear to be related to increased oxidative
stress accompanied by lower plasma ascorbic acid status. Thus, smokers, due to increased oxidative stress, have an increased need for both α-T and ascorbic acid.
3.2. INTRODUCTION

Nearly 50 million Americans smoke cigarettes (Karnath, 2002). The adverse health consequences of smoking have been largely attributed to the abundance of reactive oxygen species (ROS) and reactive nitrogen species (RNS) that readily react with various biomolecules. In fact, a single puff of cigarette smoke contains $10^{14-15}$ reactive oxygen species, ~500 ppm nitric oxide (NO) and other reactive nitrogen oxides (Pryor, 1997). In addition to the primary stress of cigarette smoke, cigarette smokers also have increased inflammatory responses which further enhance their oxidative stress (Alberg, 2002; Brown et al., 1997).

It is likely that increased oxidative stress increases dietary antioxidant utilization. Indeed, cigarette smokers have higher dietary ascorbic acid requirements than nonsmokers (Institute of Medicine (U.S.). Panel on Dietary Antioxidants and Related Compounds., 2000). However, research to date has been unable to accurately define dietary requirements for vitamin E or to determine whether cigarette smoking or other oxidative stresses increase these requirements. As a result, the 2000 Recommended Dietary Allowance for vitamin E do not consider the impact of oxidative stress on vitamin E requirement. In addition, these recommendations are based largely on the in vitro assessment of hemolysis after treatment with peroxide in erythrocytes obtained from experimentally-caused vitamin E-deficiency in men (Horwitt et al., 1963; Horwitt et al., 1956b; Institute of Medicine (U.S.). Panel on Dietary Antioxidants and Related Compounds., 2000).
In vitro studies (Eiserich et al., 1995; Frei et al., 1991; Handelman et al., 1996) have indicated that cigarette smoke exposure depletes plasma α-tocopherol (α-T), while in humans trials (al Senaidy et al., 1997; Dietrich et al., 2003; Leonard et al., 2003; Malila et al., 2002; Rust et al., 2001) α-T status is often not reported to differ between smokers and nonsmokers. However, with the use of deuterium-labeled Ts (Acuff et al., 1994; Burton et al., 1990a; Burton et al., 1998; Traber et al., 1990a; Traber et al., 1994b), investigations can be conducted to determine the biokinetics, bioavailability, and metabolism of vitamin E more precisely and independently of dietary Ts. Unfortunately, previous attempts to characterize plasma α-T biokinetics in cigarette smokers using deuterated tocopherols have not been entirely successful. The first attempt (Munro et al., 1997) supplemented smokers and nonsmokers on a single occasion with deuterated α-Ts then collected blood samples at 6, 12, and 27 h following the supplement. While smokers had lower plasma deuterated α-T concentrations at each of the time points studied, it could not be elucidated whether these differences were due to differences in α-T absorption or due to its faster plasma clearance. In order to further clarify these differences, a trial by other investigators (Traber et al., 2001) supplemented smokers and nonsmokers with deuterated α-Ts for seven days and collected fasting blood samples on select days up to day 21 post-supplementation to determine differences in disappearance rates after both groups achieved similar plasma deuterated α-T concentrations. While smokers had a faster rate of deuterated α-T disappearance, this finding was not statistically significant likely because the investigation was underpowered.

Measuring α-T concentrations in plasma and tissues has been long used to assess vitamin E status in humans. Plasma or urinary α-T metabolite, (α-CEHC; α-carboxy-ethyl-hydroxy-chroman) concentrations, however, may be another useful
biomarker to assess vitamin E status. Identified as a non-oxidation product, α-CEHC is reported to be synthesized by hepatocytes via a cytochrome P450 dependent pathway, conjugated with glucuronide or sulfate and then excreted into the urine (Birringer et al., 2001; Sontag and Parker, 2002). In humans, it appears that α-CEHC is undetectable in urine unless subjects are supplemented with sufficient α-T to surpass a plasma α-T threshold of 30-40 µM (Schuelke et al., 2000). These data suggest that metabolism occurs when adequate or excessive hepatic α-T concentrations have been achieved.

In this investigation, we hypothesized that the higher magnitude of oxidative stress experienced by cigarette smokers compared with nonsmokers would lead to more rapid plasma depletion of deuterium-labeled α-T and to decreased urinary α-CEHC concentrations. To test this hypothesis, we supplemented cigarette smokers and nonsmokers with deuterium labeled α-T and measured the disappearance of plasma α-T and appearance of urinary α-CHEC using liquid chromatography/mass spectrometry and gas chromatography/mass spectrometry, respectively.
3.3. METHODS AND MATERIALS

Study Participants

The protocol for this study was approved by the Institutional Review Board at Oregon State University and all participants provided written consent prior to enrollment. Healthy, normolipidemic volunteers (n = 20; non-smokers, n = 10; smokers, n = 10) were selected for this study on the basis of age (18-35 y), non-nutritional supplement use (> 6 months) and exercise status (< 5 h/w of aerobic activity). Participant characteristics are shown in Table 3.1. Non-smokers (n = 6 men, 4 women) were selected on the basis that they have never smoked and did not reside with a smoker. Smokers (n = 6 men, 4 women) were selected if they smoked > 10 cigarettes/day. Cotinine, the metabolite of nicotine, was measured using a radioimmunoassay (Diagnostics Products Corp, CA). A urinary cotinine concentration of > 500 ng/mL, as suggested by the manufacturer, was used as a cutoff to confirm smoking status.

To verify participants’ health status prior to enrollment in the study, a complete chemistry profile (Table 3.2) was performed at Good Samaritan Regional Medical Center (Corvallis, OR). Additionally, hemoglobin was measured on whole blood using a kit according to manufacturer’s instructions (Sigma Diagnostics; Procedure No. 525). Blood hematocrit was measured following 5 min of centrifugation (Statspin; Norwood, MA).
**Dietary Analysis**

In order to control for potential confounders with respect to differences in dietary nutrient consumption, all participants completed a three-day food record (two weekdays and one weekend day) during the investigation. Dietary intakes were analyzed using Food Processor (version 7.9; Salem, OR).

**Deuterated α-Tocopherol**

Capsules containing RRR-α-5-(CD₃)- and all rac-α-5,7-(CD₃)₂ tocopheryl acetates (d₃-RRR-α-TAc and d₆-all rac-α-TAc, respectively) were a gift from the Natural Source Vitamin E Association (NSVEA), and were synthesized by Eastman Kodak, Rochester, NY. The d₃-RRR- and d₆-all rac-α-TAc were encapsulated in a gelatin capsule as nominal mixtures 1:1 in 150 mg quantities. The molar ratio of d₃-RRR- to d₆-all rac-α-T was determined to be 0.98 (Traber et al., 1998).

**Study Protocol**

On six consecutive evenings, participants ingested a deuterated α-T supplement (75 mg each of d₃-RRR-α-TAc and d₆-all rac-α-TAc) immediately following a standard meal. On average, this meal contained 1143 kcal (43% carbohydrate, 17% protein, 41% fat), 35 mg ascorbic acid, and 2.7 mg α-T.

Blood samples were obtained after an overnight fast (10-12 h) on d -6, -5, -4, -3, -2, -1, 0, 1, 2, 3, 4, 5, 6, 8, 10, 13, 15, 17 (negative days denote supplementation period). Blood was obtained from the antecubital vein into blood collection tubes (Vacutainer, Becton Dickinson, Franklin Lakes, NJ) containing 0.05 mL 15% K₃ EDTA or sodium heparin. Smokers were asked to refrain from smoking for 1 h prior to blood collection to alleviate the transient oxidative stress effects. Urine was collected for 24 h on three occasions: prior to supplementation (d -6), d 0, and 17.
After total urine volume was determined, aliquots were collected and stored at -40ºC until analysis.

**Blood Sample Handling**

Blood tubes with additives were kept on ice for less than 30 minutes prior to centrifugation. Plasma was separated by centrifugation (15 m at 500 x g, 4ºC; Beckman TJ-6, Paola Alto, CA), aliquoted into cryovials, snap frozen in liquid nitrogen, and then stored at -80ºC until analysis. After plasma was separated, an aliquot was acidified (1:1) with 10% PCA (perchloric acid) containing 1 mM DTPA (diethylenetriaminepentaacetic acid). This sample was then centrifuged (5 min, 15,000 x g, 4ºC; Eppendorf Centrifuge 5415R, Hamburg, Germany), the supernatant was removed, snap frozen, and stored at -80ºC for future analysis of ascorbic and uric acid.

**Materials**

PCA and HPLC-grade methanol were obtained from Fisher (Fair Lawn, NJ). The following were obtained from Sigma-Aldrich (St. Louis, MO): ascorbic acid, butylated hydroxy toluene (BHT), DTPA, FeCl₃, phosphate buffer saline, potassium hydroxide (KOH), potassium phosphate trihydrate, TPTZ (2, 4, 6-tri(2-pyridil)-s-triazine), and trolox. Chromatography pairing reagent, Q12 (1-dodecyltriethyl-ammonium phosphate), was purchased from Regis (Morton Grove, IL). Standards including unlabeled (d₀), d₆-RRR- and d₃-all rac-α-TAc and unlabeled d₀-γ-T were gifts from Dr. James Clark of Cognis Nutrition and Health, LaGrange, IL. *all rac-α-5,7,8-(CD₃)₃ TAc* (d₀-*all rac-α-TAc*) was provided by Dr. Carolyn Good of General Mills and was synthesized by Isotec Inc. (Miamisburg, OH). The isotopic purity was found to be 88.4% d₉ and the remainder d₈.
**Tocopherol Analysis**

Labeled and unlabeled Ts were extracted according to procedures previously described (Podda et al., 1996b) and were analyzed using a LC/MS (liquid chromatography/mass spectrometer) consisted of a Waters 2690 Separations Module (Milford, MA) and a ZQ 2000 single-quadrupole mass spectrometer (Micromass Manchester, England), as previously described (Leonard et al., 2003). The LC/MS was equipped with an APCI (atmospheric pressure chemical ionization) probe set to the negative ionization mode. Mass-to-charge \((m/z)\) ratios were obtained as follows: \(d_0-\alpha-T, m/z\ 429.4\), \(d_3-\alpha-T, m/z\ 432.3\), \(d_6-\alpha-T, m/z\ 435.4\), \(d_9-\alpha-T, m/z\ 438.4\) and \(d_0-\gamma-T, m/z\ 415.4\). Calibration curves were prepared using the authentic standards and all \(rac-\alpha-5,7,8-(CD_3)_3-T\) (\(d_9-\alpha-T\)) was used as the internal standard. Concentrations of the T standards were determined spectrophotometrically (Beckman DU Series 600, Fullerton, CA) using: \(\varepsilon_{292\text{ nm}^{\text{EtOH}} = 3,270\ \text{M}^{-1}\ cm^{-1}}\) for \(\alpha-T\) and \(\varepsilon_{298\text{ nm}^{\text{EtOH}} = 3,810\ \text{M}^{-1}\ cm^{-1}}\) for \(\gamma-T\).

**Analysis of CEHCs**

Urinary deuterium labeled and unlabeled T metabolites (\(\alpha-\) and \(\gamma-\)CEHCs) were measured by gas chromatography/mass spectrometry (GC/MS) according to a previously described method (Galli et al., 2002). The GC/MS consisted of an Aglient 6890 GC coupled with an Aglient 5973N MSD and analyte concentrations were determined utilizing electron impact ionization. In short, the urinary metabolites were extracted from 5 ml of urine, derivatized with BSTFA (N,O-bis (trimethylsilyl) trifluoroacetamide) and 1% trimethylchlorosilane, dried under nitrogen, and resuspended in hexane prior to injection. Samples volumes of 1 \(\mu L\) were injected onto the column with a HP 7683 auto-injector. Separations were performed on a DB-5MS column (J & W Scientific, 30 m x 2.5 mm i.d; 2.5 \(\mu m\) film thickness) with
helium as carrier gas. Quantification was performed by comparison to known amounts of an added internal standard (Trolox).

**Ascorbic Acid and Uric Acid Analysis**

Ascorbic and uric acid were measured by HPLC-EC as previously described (Frei et al., 1989a). Standards of ascorbic acid were prepared fresh daily and verified spectrophotometrically using the $\varepsilon_{265nm}$ of 14,500 M$^{-1}$ cm$^{-1}$. Prepared uric acid standard was purchased from Pointe Scientific, Inc (Lincoln Park, MI).

**Ferritin Reducing Ability of Plasma (FRAP) Determination**

To assess plasma antioxidant capacity, FRAP values were measured according to the methods of Benzie and Strain (Benzie and Strain, 1996). In brief, 40 µL of plasma (diluted 1:4) was mixed on a 96-well plate with 300 µL of freshly prepared FRAP reagent (25 ml 300 mM sodium acetate buffer, 2.5 ml 10 mM TPTZ and 2.5 ml 20 mM FeCl$_3$). Samples were incubated for 15 min at 37ºC prior to reading at 550 nm on Spectramax 190 microplate reader (Molecular Devices; Sunnyvale, CA). FRAP values were calculated using trolox as a standard.

**Lipid Analysis**

Plasma $F_2\alpha$-isoprostanes were measured by GC/MS as previously described (Morrow and Roberts, 1994). Total cholesterol and triglycerides were measured using kits obtained from Sigma Diagnostics (Procedure No. 343 and 401, respectively) and performed in accordance with manufacturer’s instructions.

**Mathematical Analysis of T Disappearance Kinetics**

The percentage of $d_3$-$\alpha$-T ($\%d_3$-$\alpha$-T = ($d_3$-$\alpha$-T/($d_0$-$\alpha$-T + $d_3$-$\alpha$-T + $d_6$-$\alpha$-T)) x 100) was calculated from the plasma T concentrations for each subject, at each time
point, and was fitted by a two-compartment model, as described in a previous investigation (Traber et al., 2001). The two compartments were assumed to have reached the same concentration at the end of six d of deuterated T supplementation. Fitting was by nonlinear least squares, assuming measurement error to have a constant coefficient of variation. Labeled T half-lives were calculated as $t_{1/2} = \ln(2)/\text{disappearance rate constant}$.

**Statistical Analysis**

The Student’s t-test was used to compare subjects’ baseline characteristics, dietary intake, $\%d_3$-$\alpha$-T rate constants, and urinary $\alpha$-CEHCs. Repeated measures ANOVA (analysis of variance) with Fisher’s post hoc test was used to analyze statistical differences over time between smokers and nonsmokers in plasma $F_{2\alpha}$-isoprostanes, triglycerides, cholesterol, FRAP, ascorbic acid, and uric acid. Data were considered statistically significant when $p < 0.05$. All data are displayed as mean ± SE unless otherwise noted.
3.4. RESULTS

Participant Characteristics and Analysis of Dietary Intakes

There were no differences at baseline between smokers and nonsmokers with respect to age, sex, height, weight, BMI (body mass index), plasma lipids (total cholesterol and triglycerides), α-T, γ-T, uric acid, or ascorbic acid concentrations (Table 3.1). Participating cigarette smokers self-reported smoking between 10-20 cigarettes per day. Urinary cotinine values were correlated with self-reported cigarettes/day ($p < 0.010; R^2 = 0.582$) among the smokers. Nonsmokers had low or no measurable urinary cotinine concentrations (Table 3.1).

Surprisingly, no differences were observed for baseline ascorbic acid between nonsmokers and smokers (60 ± 7 µM compared to 55 ± 6 µM). Since it is often reported that cigarette smokers have lower plasma ascorbic acid status than nonsmokers (Alberg, 2002; Faruque et al., 1995; Marangon et al., 1998; Mezzetti et al., 1995) these data suggest that the cohort of smokers in this study may not be a representative population of cigarette smokers. It is possible that these similarities in plasma ascorbic acid status could be due to a seasonal effect (Cooney et al., 1995a; Lenton et al., 2000; Woodhouse and Khaw, 2000) since this study was conducted in early autumn. However, while these results were unplanned, the similarities in ascorbic acid status may help to isolate the effects of cigarette smoking on α-T disappearance kinetics.
None of the participants took nutritional supplements for at least 6-months prior to our study. Smokers and nonsmokers had similar dietary intakes of the nutrients analyzed (Table 3.3).

**Plasma Ascorbic and Uric Acid**

Throughout the investigation (d -6 to 17), nonsmokers and smokers had similar plasma ascorbic acid concentrations (Table 3.4; main effect \( p = 0.676 \)). However, a correlation was observed between plasma ascorbic acid concentrations and dietary ascorbic acid consumption obtained from the three day food records (\( p = 0.011; R^2 = 0.324 \)). Uric acid concentrations, another major plasma antioxidant, were also similar among the groups (Table 3.4; main effect \( p = 0.763 \)) throughout the investigation.

**FRAP**

FRAP is a measure of total antioxidant protection in plasma and is generally correlated mainly with uric acid concentration and to a lesser extent with ascorbic acid concentrations (Benzie and Strain, 1996). Smokers compared to nonsmokers had lower FRAP values (Table 3.4) throughout the investigation suggesting that smokers had lower plasma antioxidant status (main effect; \( p = 0.037 \)). Mean FRAP values throughout the study correlated positively with mean uric acid status (\( p = 0.001; R^2 = 0.456 \)), but not with plasma ascorbic acid (\( p = 0.083; R^2 = 0.158 \)).

**Plasma Isoprostanes**

To assess oxidative stress, \( F_{2\alpha} \)-isoprostanes were measured on selected days throughout the study. Cigarette smokers, on average, had \( F_{2\alpha} \)-isoprostanes that were \(~42\%\) higher than nonsmokers throughout the entire study (Table 3.4; main effect; \( p = 0.019 \)). During the supplementation period, smokers’ plasma \( F_{2\alpha} \)-isoprostanes
decreased slightly (~13%) from d-6 (47 ± 5 pg/mL) to d 0 (41 ± 4 pg/mL), but these differences did not reach statistical significance (p = 0.132).

**Tocopherols**

Prior to supplementation, smokers and nonsmokers had similar plasma unlabeled T concentrations (Table 3.1). After six d of supplementation with 75 mg each of d3-RRR- and d6-all rac-α-TAcs, plasma total α-Ts (sum of d0-, d3- and d6-α-T) more than doubled (p < 0.0001) in nonsmokers (from 15.3 ± 2.8 μM at baseline to 35.5 ± 5.8 μM on d 0) and in smokers (from 14.6 ± 3.8 μM at baseline to 32.1 ± 10.2 on d 0). Both groups had similar d0-, d3-, d6-α-T, and total α-T concentrations on day 0 (Figure 3.1).

In both groups, plasma d0-α-T concentrations decreased (p < 0.0001) ~19% in response to deuterated α-T supplementation from baseline (14.9 ± 0.7 μM) to d 0 (12.1 ± 0.8 μM). Similarly, plasma γ-T concentrations decreased (p = 0.0011) ~38% in both groups (from 1.6 ± 0.1 μM at baseline to 1.0 ± 0.1 μM at d 0).

At day 0, plasma d3-α-T concentrations in the nonsmokers and smokers (13.6 ± 1.2 μM vs. 12.2 ± 1.5 μM, respectively) peaked similarly suggesting that both groups achieved similar plasma concentrations as a result of 6-d supplementation (Figure 3.1). Likewise, plasma d6-α-T concentrations were similar in nonsmokers and smokers (8.8 ± 0.8 μM vs. 8.6 ± 0.9 μM). The ratios of d3-α-T to d6-α-T in the plasma were 1.5 ± 0.1 during the supplementation period and subsequently 1.9 ± 0.1 post-supplementation (d 0-17). These findings are similar to other human trials (Acuff et al., 1994; Burton et al., 1998; Traber et al., 1994a) that have used deuterated RRR- and all rac-Ts and therefore the subsequent results and discussion of α-T disappearance kinetics will be limited to d3-α-T for simplicity.
After 6-d deuterated α-T supplementation, peak %d₃-α-T on day 0 did not differ between groups (Figure 3.1) and d₃-α-T represented ~40% of total plasma α-T. Mathematical modeling of the disappearance kinetics was performed on the %d₃-α-T from d 0 through 17 and data fitting from a representative smoker and nonsmoker are shown in Figure 3.2. The fractional %d₃-α-T disappearance rates in cigarette smokers (0.215 ± 0.001) were ~13% greater than nonsmokers (0.191 ± 0.001 pools/day; p < 0.05; Figure 3.3). Correspondingly, %d₃-α-T half-lives were shorter in cigarette smokers (79.3 ± 4.1) compared with nonsmokers (88.8 ± 3.8 h; p < 0.05).

Consistent with these results, smokers’ plasma d₃-α-T concentrations on day 17 were lower than that of the nonsmokers (p < 0.05). Similarly, smokers’ d₀-α-T concentrations were lower (p < 0.05) than those of nonsmokers by the end of the study. Despite this finding, there were no observable differences between smokers and nonsmokers in the d₀-α-T concentrations prior to supplementation and both had similar α-T intakes as assessed by dietary food records during the study.

It has been suggested that plasma T concentrations should be adjusted for circulating lipid concentrations (Gross et al., 2003). However, in this investigation no changes were observed in plasma total cholesterol or triglycerides on any of the study days (Table 3.4). Therefore, labeled and unlabeled T concentrations were not adjusted for total lipids.

**Urinary Tocopherol Metabolite**

The metabolite of α-T, α-CEHC, was measured on three occasions during the investigation from 24-hour urine collections: baseline (d -6), after 6 d of supplementation (d 0), and on the last day of the study (d 17). Given the limited data on α-T metabolism, we hypothesized that metabolite concentrations might serve as marker for α-T status. As shown in Figure 3.4, there were no differences between
smokers and nonsmokers with respect to unlabeled and labeled α-CEHCs at baseline or on day 0, suggesting that both groups had similar α-T status. Furthermore, the ratio of $d_6$-α-CEHC:$d_3$-α-CEHC on day 0 was $1.7 \pm 0.1$ for both groups, indicating a greater conversion of $d_6$-all rac-α-T to α-CEHC compared with $d_3$-α-RRR-T.

On day 17, seven of 10 nonsmokers, but only five of 10 smokers, had detectable urinary deuterated α-CEHCs. From the analysis of these available data it was observed that smokers excreted less $d_0$- ($p < 0.05$), $d_3$- ($p < 0.05$), and $d_6$-α-CEHCs ($p = 0.06$) than did nonsmokers. Analysis of total plasma α-Ts and urinary α-CEHCs between the smokers and nonsmokers also demonstrated that total plasma α-Ts and urinary α-CEHCs were lower among the smokers only on d 17 (Table 3.5). These data collectively supported the notion that smokers had lower α-T status at the end of the study.

**Interaction Between α-T Disappearance and Ascorbic Acid and Uric Acid**

Ascorbic acid and α-T have been shown to interact under *in vitro* conditions (Bisby and Parker, 1995). Therefore we calculated correlations between the %$d_3$-α-T fractional disappearance rate and mean plasma ascorbic acid concentrations obtained from days 0-17. Mean ascorbic acid concentrations during this period were similar for the nonsmokers (52 ± 4 µM) and smokers (47 ± 6 µM). The %$d_3$-α-T fractional disappearance rates were correlated with plasma ascorbic acid in the cigarette smokers ($p = 0.021$; $R^2 = 0.509$; Figure 3.5), but not in the nonsmokers ($p = 0.505$; $R^2 = 0.057$). This observation suggests that the faster α-T disappearance rates were related to lower ascorbic acid concentrations only in the presence of apparent oxidative stress observed in the smokers.

A similar analysis was performed using uric acid since this water-soluble antioxidant is present in the plasma in significantly higher concentrations than
ascorbic acid. No significant correlations between the α-T disappearance rates and mean uric acid concentrations were observed either for the nonsmokers ($p = 0.075; R^2 = 0.342$) or the smokers ($p = 0.446; R^2 = 0.074$).
3.5. DISCUSSION

Oxidative stress caused by cigarette smoking resulted in increased α-T disappearance rates (Figure 3.3). Smokers and nonsmokers were supplemented with deuterium labeled α-T (75 mg each d$_3$-RRR and d$_6$-all rac-α-TAc) for six days, a length of time that was sufficient for both groups to achieve similar plasma deuterated α-T concentrations (Figure 3.1). Once supplementation ceased, the %d$_3$-α-T fractional disappearance rates and half-lives of the smokers were ~13% greater and ~11% shorter, respectively (Figure 3.3). As would be expected from these data, day 17 smokers’ plasma d$_3$-α-T concentrations were lower than those of nonsmokers.

In cigarette smokers, but not in nonsmokers, α-T disappearance rates were significantly correlated with plasma ascorbic acid concentrations (Figure 3.5). By linear regression analysis of the α-T disappearance rate and the mean plasma ascorbic acid concentration for each subject, we estimated that cigarette smokers would require a plasma ascorbic acid concentration of 64.2 µM in order to produce similar α-T fractional disappearance kinetics as the nonsmokers (0.191 pools/day). This calculated estimate is ~37% higher than the observed mean plasma ascorbic acid concentration of 47 µM in the smokers. Since dietary ascorbic acid was linearly correlated with plasma ascorbic acid in the smokers ($p = 0.005; R^2 = 0.697$), we further calculated that the smokers would require a total dietary ascorbic acid intake of 116 mg to achieve a plasma concentration of 64.2 µM. On average, this estimate would provide smokers with an additional 43 mg/day of ascorbic acid above their reported dietary consumption (Table 3.3). However, these calculations are
comparable to the current dietary ascorbic acid recommendations which suggest that smokers consume an additional 35 mg/day of ascorbic acid above the recommendations for nonsmokers (90 and 75 mg/day for men and women, respectively) (Institute of Medicine (U.S.). Panel on Dietary Antioxidants and Related Compounds., 2000; Kallner et al., 1981).

Although $\alpha$-T disappearance rates in smokers were correlated with ascorbic acid concentrations (Figure 3.5), and smokers and nonsmokers had similar average plasma ascorbic acid concentrations (Table 3.1), it is apparent that the range of ascorbic acid concentrations was greater in the smokers and that low ascorbic acid concentrations were observed in those smokers with the fastest $\alpha$-T disappearance rates. These data suggest that both low plasma ascorbic acid concentrations and oxidative stress are necessary to negatively influence the $\alpha$-T disappearance kinetics. To our knowledge, this is the first time that this interaction between ascorbic acid and $\alpha$-T has been demonstrated in humans. Previously, guinea pigs fed two concentrations of deuterium labeled $\alpha$-T and three concentrations of ascorbic acid did not shown an interaction between these nutrients (Burton et al., 1990b). However, no oxidative stress was applied to the guinea pigs in that investigation. Alternatively, in vitro studies conducted with H4IIE liver cells (Huang and May, 2003) or erythrocytes (May et al., 1998) subjected to oxidative stress sufficient enough to cause lipid peroxidation demonstrated a sparing effect of $\alpha$-T when cells were treated with ascorbic acid. Similarly, in another in vitro investigation it was determined that the $\alpha$-tocopheroxyl radical formed within micellar and bilayer membrane systems could be effectively recycled by ascorbic acid found within the aqueous phase (Bisby and Parker, 1995).

The results of our investigation demonstrated that smokers have modestly higher requirements of $\alpha$-T than nonsmokers. However, it should not be overlooked
that the cohort of participants in this investigation were young (18-25 years) with a limited history (2-5 years) and frequency (10-20 cigarettes/day) of smoking. While it can not be ascertained from the available data in this investigation, we can only speculate that these differences would be greater among older individuals with a greater history and frequency of smoking since it is believed that “aging” in itself is an oxidative stress (Ames et al., 1993).

We measured plasma isoprostanes concentrations as an index of oxidative stress. Smokers had higher plasma isoprostanes concentrations than nonsmokers throughout the entire study. While, there was a modest reduction in circulating isoprostanes during deuterated α-TAc supplementation (d -6 to d 0), these changes in isoprostanes were not statistically significant. Both human (Huang et al., 2002; Kaikkonen et al., 2001) and animal (Willcox et al., 2003) investigations have demonstrated that reductions in circulating isoprostanes by α-T supplementation require a much longer intervention or the administration of a higher α-T dose.

Lastly, the urinary metabolite of α-T (α-CEHC) was lower in smokers compared to nonsmokers on d 17 suggesting that less α-T metabolism occurred because there was less “excess” α-T available for degradation. Collectively, these data suggest that cigarette smokers due to their increased oxidative stress require higher dietary α-T intakes to maintain similar plasma α-T concentrations as nonsmokers. The relationship in smokers between faster α-T disappearance and lower plasma ascorbic acid concentrations warrants further assessment to determine if intervention with ascorbic acid supplementation can modify α-T disappearance kinetics in cigarette smokers.
3.6. ACKNOWLEDGMENTS

The subjects receive our thanks for their cooperation. The authors are grateful to the Natural Source Vitamin E Association for providing the capsules of deuterium labeled α-T as well as partial support for the purchase of the LC/MS. Special thanks to Scott Leonard of the Linus Pauling Institute for his technical assistance of LC/MS T analysis. We would also like to thank Wendy McMahan of the Montine Lab for performing the plasma isoprostane analysis. Financial support for this work was provided by grants to MGT (NIH DK59576) and TJM (AG05144 and AG16835).
<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Nonsmokers (n = 10)</th>
<th>Smokers (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>19.5 ± 1.5</td>
<td>21.0 ± 3.1</td>
</tr>
<tr>
<td>Height (m)</td>
<td>1.69 ± 0.15</td>
<td>1.76 ± 0.13</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>63.6 ± 14.4</td>
<td>68.0 ± 9.0</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>22.2 ± 3.2</td>
<td>22.0 ± 2.2</td>
</tr>
<tr>
<td>Nutrition supplements</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cigarettes/day</td>
<td>-</td>
<td>10 – 20</td>
</tr>
<tr>
<td>Cotinine (ng/mL)</td>
<td>34 ± 13</td>
<td>2345 ± 1555</td>
</tr>
<tr>
<td>α-T (µM)</td>
<td>15.3 ± 2.8</td>
<td>14.6 ± 3.8</td>
</tr>
<tr>
<td>γ-T (µM)</td>
<td>1.63 ± 0.6</td>
<td>1.56 ± 0.7</td>
</tr>
<tr>
<td>Ascorbic Acid (µM)</td>
<td>60 ± 22</td>
<td>55 ± 19</td>
</tr>
<tr>
<td>Uric Acid (µM)</td>
<td>273 ± 57</td>
<td>293 ± 47</td>
</tr>
<tr>
<td>Total Cholesterol (mmol/L)</td>
<td>4.34 ± 0.44</td>
<td>3.87 ± 0.96</td>
</tr>
<tr>
<td>HDL (mmol/L)</td>
<td>1.37 ± 0.17</td>
<td>1.45 ± 0.35</td>
</tr>
<tr>
<td>LDL (mmol/L)</td>
<td>2.42 ± 0.40</td>
<td>1.91 ± 0.64</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>1.19 ± 0.55</td>
<td>1.11 ± 0.43</td>
</tr>
</tbody>
</table>

**Table 3.1. Participant characteristics at baseline**
Baseline characteristics (mean ± SD) of nonsmokers and smokers were similar ($p > 0.05$) in all respects except for urinary cotinine and cigarettes smoked.
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Normal Range</th>
<th>Nonsmokers (n = 10)</th>
<th>Smokers (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium (mEq/L)</td>
<td>135 - 145</td>
<td>142 ± 2</td>
<td>142 ± 1</td>
</tr>
<tr>
<td>Potassium (mEq/L)</td>
<td>3.5 - 5.1</td>
<td>4.1 ± 0.2</td>
<td>4.6 ± 0.6</td>
</tr>
<tr>
<td>Chloride (mEq/L)</td>
<td>100 - 111</td>
<td>104 ± 1</td>
<td>103 ± 2</td>
</tr>
<tr>
<td>Bicarbonate (mEq/L)</td>
<td>22 - 30</td>
<td>25 ± 2</td>
<td>26 ± 3</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>70 - 105</td>
<td>83 ± 6</td>
<td>81 ± 11</td>
</tr>
<tr>
<td>BUN (mg/dL)</td>
<td>6 - 19</td>
<td>13 ± 3</td>
<td>13 ± 4</td>
</tr>
<tr>
<td>Creatinine (mg/dL)</td>
<td>0.4 - 1.1</td>
<td>0.9 ± 0.1</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>BUN/creatinine ratio</td>
<td>6 - 30</td>
<td>16 ± 3</td>
<td>17 ± 4</td>
</tr>
<tr>
<td>Calcium (mg/dL)</td>
<td>8.4 - 10.2</td>
<td>9.8 ± 0.5</td>
<td>10.1 ± 0.4</td>
</tr>
<tr>
<td>Phosphorus (mg/dL)</td>
<td>2.7 - 4.5</td>
<td>3.8 ± 0.5</td>
<td>4.3 ± 0.7</td>
</tr>
<tr>
<td>Total protein (g/dL)</td>
<td>6.4 - 8.3</td>
<td>7.5 ± 0.2</td>
<td>7.3 ± 0.5</td>
</tr>
<tr>
<td>Albumin (g/dL)</td>
<td>3.4 - 5.0</td>
<td>4.4 ± 0.4</td>
<td>4.5 ± 0.3</td>
</tr>
<tr>
<td>SGOT (U/L)</td>
<td>0 - 31</td>
<td>20 ± 3</td>
<td>25 ± 19.2</td>
</tr>
<tr>
<td>LDH (U/L)</td>
<td>94 - 250</td>
<td>157 ± 11</td>
<td>201 ± 121</td>
</tr>
<tr>
<td>SGPT (U/L)</td>
<td>0 - 31</td>
<td>16 ± 6</td>
<td>18 ± 6</td>
</tr>
<tr>
<td>Alkaline phosphatase (U/L)</td>
<td>39 - 117</td>
<td>73 ± 24</td>
<td>80 ± 16</td>
</tr>
<tr>
<td>Gamma GT (U/L)</td>
<td>7 - 33</td>
<td>17 ± 9</td>
<td>14 ± 5</td>
</tr>
<tr>
<td>Total bilirubin (mg/dL)</td>
<td>0.0 - 1.0</td>
<td>0.6 ± 0.3</td>
<td>0.6 ± 0.2</td>
</tr>
<tr>
<td>Globulin (g/dL)</td>
<td>2.3 - 3.5</td>
<td>3.1 ± 0.4</td>
<td>2.8 ± 0.3</td>
</tr>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>13.0 - 16.8</td>
<td>16.0 ± 1.4</td>
<td>15.4 ± 1.2</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>38 - 49</td>
<td>45.5 ± 4.1</td>
<td>45.9 ± 3.4</td>
</tr>
</tbody>
</table>

Table 3.2. Subjects' blood chemistry and hematologic values at screening
Nonsmokers and smokers had similar blood chemistries (mean ± SD) and were within the normal reference range (data from Good Samaritan Hospital).
### Table 3.3. Analysis of dietary intake of nonsmokers and smokers.

Three day dietary intakes (mean ± SD) in nonsmokers and smokers. No statistical differences were observed between groups for any nutrient. Dietary ascorbic acid intakes and plasma ascorbic acid concentrations were correlated ($p = 0.011$; $R^2 = 0.323$).

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Nonsmokers</th>
<th>Smokers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy (kcal)</td>
<td>2703 ± 498</td>
<td>2807 ± 773</td>
</tr>
<tr>
<td>Carbohydrate (%)</td>
<td>50.5 ± 4.9</td>
<td>52.5 ± 2.2</td>
</tr>
<tr>
<td>Protein (%)</td>
<td>15.4 ± 1.5</td>
<td>14.1 ± 2.3</td>
</tr>
<tr>
<td>Fat (%)</td>
<td>35.0 ± 3.9</td>
<td>31.9 ± 5.3</td>
</tr>
<tr>
<td>Ascorbic Acid (mg)</td>
<td>77.6 ± 25.3</td>
<td>73.4 ± 36.1</td>
</tr>
<tr>
<td>Vitamin E ($\alpha$-T mg)</td>
<td>5.5 ± 1.1</td>
<td>5.3 ± 2.2</td>
</tr>
<tr>
<td></td>
<td>Nonsmokers</td>
<td>Smokers</td>
</tr>
<tr>
<td>--------------------------</td>
<td>------------</td>
<td>----------</td>
</tr>
<tr>
<td>Cholesterol (mmol/L)</td>
<td>4.36 ± 0.55</td>
<td>3.76 ± 0.95</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>0.96 ± 0.34</td>
<td>0.75 ± 0.28</td>
</tr>
<tr>
<td>Ascorbic Acid (µM)</td>
<td>51 ± 13</td>
<td>48 ± 18</td>
</tr>
<tr>
<td>Uric Acid (µM)</td>
<td>284 ± 42</td>
<td>278 ± 51</td>
</tr>
<tr>
<td>FRAP (µM)</td>
<td>635 ± 60</td>
<td>559 ± 88</td>
</tr>
<tr>
<td>F₂-Isoprostanes (pg/ml)</td>
<td>32 ± 5.9</td>
<td>46 ± 15.7</td>
</tr>
</tbody>
</table>

Table 3.4. Plasma concentrations of lipids and antioxidant status markers throughout the entire investigation (d -6 to d 17) for nonsmokers and smokers. Shown are mean ± SD for each of the plasma markers. Smokers had significantly higher F₂-isoprostanes and lower FRAP, but no differences were observed with regard to ascorbic acid, uric acid, cholesterol or triglycerides.
### Table 3.5. Comparison between total plasma α-tocopherol and total urinary α-CEHC

Supplementation with labeled α-T resulted in a doubling (mean ± SD) of total α-tocopherol (d₀- + d₃- + d₆-α-T) in both nonsmokers and smokers (p < 0.0001). In addition, supplementation resulted in a 5.6 and 4.3 fold increase (mean ± SD) in total urinary α-CEHC (d₀- + d₃- + d₆-α-CEHC) excretion in the nonsmokers and smokers, respectively. aOn day 17, smokers had lower total α-tocopherol (p = 0.011) and total α-CEHC (p = 0.037) compared to the nonsmokers.

<table>
<thead>
<tr>
<th></th>
<th>Non-smokers</th>
<th>Smokers</th>
<th>Non-smokers</th>
<th>Smokers</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Day -6</strong></td>
<td>15.3 ± 2.8</td>
<td>14.6 ± 3.8</td>
<td>540 ± 560</td>
<td>570 ± 834</td>
</tr>
<tr>
<td><strong>Day 0</strong></td>
<td>35.5 ± 5.6</td>
<td>32.1 ± 10.2</td>
<td>3039 ± 2889</td>
<td>2383 ± 1068</td>
</tr>
<tr>
<td><strong>Day 17a</strong></td>
<td>19.8 ± 4.0</td>
<td>15.3 ± 3.9</td>
<td>859 ± 850</td>
<td>262 ± 251</td>
</tr>
</tbody>
</table>
Figure 3.1. Plasma d\textsubscript{0}-, d\textsubscript{3}-, and d\textsubscript{6}-\textalpha-T time course in smokers and nonsmokers

Participants were supplemented for six d (denoted by negative sign) with 75 mg each d\textsubscript{3}-RRR\textsuperscript{-} and d\textsubscript{6}-\textit{all rac}-\textalpha-TAc following a standard meal. All blood samples were collected in the morning after fasting for ~12 h. Shown are the d\textsubscript{0}, d\textsubscript{3}, d\textsubscript{6}-\textalpha-T concentrations (upper panels) and percentages (lower panels) in nonsmokers (left panels) and smokers (right panels).
Figure 3.2. Curve fit of \%d_3\-\(\alpha\)-T from a representative smoker and nonsmoker. Shown is the modeled curve fit and the actual data as \%d_3\-\(\alpha\)-T = (d_3\-\(\alpha\)-T / (d_0\-\(\alpha\)-T + d_3\-\(\alpha\)-T + d_6\-\(\alpha\)-T)) x 100) from day 0 to day 17 in a smoker (upper panel) and a nonsmoker (lower panel).
Figure 3.3. Fractional disappearance rates of %d3-α-T in smokers and nonsmokers
Smokers had a faster rates of %d3-α-T disappearance ($p < 0.05$).
Figure 3.4. Urinary labeled and unlabeled α-CEHC excretion
The urinary metabolite, α-CEHC, did not differ between smokers and nonsmokers prior to supplementation or at day 0. On day 17, smokers excreted less d₀- ($p < 0.05$), d₃- ($p < 0.05$), and d₆-α-CEHCs ($p = 0.06$) than did non-smokers. On d-6 and 0, n=10 subjects in each group, while on d 17, only 7 of 10 nonsmokers, and 5 of 10 smokers had detectable urinary deuterated α-CEHCs.
Figure 3.5. Fractional disappearance rate of $\%d_3$-$\alpha$-T and plasma ascorbic acid are correlated in smokers, but not in nonsmokers

Linear regression was performed to determine the relationship between the fractional $\%d_3$-$\alpha$-T disappearance rates and plasma ascorbic acid concentrations. As shown in the lower panel, no significant relationship was found among the nonsmokers ($p = 0.505; R^2 = 0.057$). However, this relationship was statistically relevant in smokers (upper panel). With decreasing plasma concentrations of ascorbic acid, the rates of plasma $d_3$-$\alpha$-T disappearance increased.
CHAPTER 4

VITAMIN C SUPPLEMENTATION PREVENTS FASTER VITAMIN E DISAPPEARANCE IN SMOKERS
4.1. SUMMARY

The effects of cigarette smoking on human vitamin E status are inconclusive. In addition, it is speculated that vitamin C can reduce vitamin E radicals, but no direct \textit{in vivo} evidence has been demonstrated. To test the hypothesis that cigarette smoking causes faster depletion of plasma vitamin E, and supplementation with ascorbic acid attenuates the depletion, we conducted a double-blind, placebo-controlled, randomized cross-over investigation to determine the effects of two-weeks supplementation with ascorbic acid (500 mg; twice daily) or placebo on plasma disappearance rates of deuterium-labeled $\alpha$- and $\gamma$-tocopherols in smokers ($n = 10$) and nonsmokers ($n = 12$). Ascorbic acid supplementation approximately doubled plasma ascorbic concentrations in smokers ($43 \pm 6$ to $97 \pm 3$ $\mu$M; $p < 0.0001$) and nonsmokers ($46 \pm 4$ to $91 \pm 4$ $\mu$M; $p < 0.0001$) relative to the placebo trial. However, ascorbic acid supplementation attenuated $\alpha$- and $\gamma$-tocopherol plasma disappearance rates by 24% ($p < 0.05$) and 30% ($p < 0.05$), respectively, among the smokers only. Likewise, only smokers’ plasma deuterium labeled $\alpha$-tocopherol ($p < 0.05$) and $\gamma$-tocopherol ($p < 0.05$) concentrations at the end of the trial (72 h) were higher when supplemented with ascorbic acid compared to placebo. The favorable changes among the smokers occurred despite no significant changes in plasma maximal ($p > 0.05$) or time of maximal ($p > 0.05$) deuterium labeled $\alpha$- and $\gamma$-tocopherol concentrations, unlabeled plasma $\alpha$- and $\gamma$-tocopherol concentrations ($p > 0.05$), or labeled or unlabeled $\alpha$- or $\gamma$-tocopherol metabolite concentrations ($p > 0.05$). Plasma F$_{2\alpha}$-isoprostanes were $\sim 34\%$ higher among smokers ($64 \pm 13$ pg/ml) compared to nonsmokers ($47 \pm 5$ pg/ml), but
these differences did not reach statistical significance ($p > 0.05$) and ascorbic acid supplementation did not reduce plasma $F_{2\alpha}$-isoprostanes in either group ($p > 0.05$), nor was it correlated with plasma $F_{2\alpha}$-isoprostanes ($p > 0.05$). In conclusion, cigarette smoking increased $\alpha$- and $\gamma$-tocopherol disappearance, suggesting that the oxidative stress from smoking oxidizes tocopherols and that plasma ascorbic acid reduces $\alpha$- and $\gamma$-tocopheroxyl radicals to nonoxidized forms thereby, decreasing vitamin E disappearance.
4.2. INTRODUCTION

Vitamin E is a lipophilic, chain-breaking antioxidant found within biological membranes that can prevent oxidative damage (Traber and Packer, 1995). It exists as a mixture of α-, β, γ-, and δ- tocopherols and tocotrienols that differ in the saturation of the phytol tail as well as the number and position of methyl groups found on the chromanol ring (Brigelius-Flohe and Traber, 1999). Vitamin E’s most well known biological function is its ability to act as a chain-breaking antioxidant that ceases the propagation of lipid peroxidation (Burton and Ingold, 1989). Thus, vitamin E acts as a peroxyl radical scavenger and is able to protect polyunsaturated fatty acids from lipid peroxidation (Burton et al., 1983). Of most biological interest are α-tocopherol and γ-tocopherol since these are the forms that are present in measurable amounts in plasma and tissues (Traber, 1999). In comparison to α-tocopherol, γ-tocopherol is the major tocopherol found in the diet but is generally found in much lesser quantities in vivo (Traber and Arai, 1999), typically accounting for 10-20% of the total tocopherols found in plasma (Traber and Kayden, 1989). This difference in biological activity has been attributed to the specificity of the hepatic α-tocopherol transfer protein since it facilitates secretion of α-tocopherol from the liver to the plasma by a mechanism that is incompletely understood (Traber, 2004).

Currently, vitamin E dietary recommendations for men and women are 15 mg/day of α-tocopherol (Institute of Medicine (U.S.). Panel on Dietary Antioxidants and Related Compounds., 2000), which is based on the in vitro assessment of peroxide-induced hemolysis of erythrocytes obtained from experimentally-caused
vitamin E-deficient men (Horwitt et al., 1963; Horwitt et al., 1956b; Institute of Medicine (U.S.). Panel on Dietary Antioxidants and Related Compounds., 2000). No further recommendations were put forth regarding the seven other vitamin E homologues because they are not converted to α-tocopherol and are poorly recognized by the α-tocopherol transfer protein found in the liver (Institute of Medicine (U.S.). Panel on Dietary Antioxidants and Related Compounds., 2000). In addition, only α-tocopherol has been shown to reverse vitamin E deficiency symptoms in humans (Gabsi et al., 2001).

Tocopherol metabolism, occurring through a hepatic cytochrome P450 dependent pathway that is incompletely understood (Birringer et al., 2001), may eventually provide investigators with an additional marker to evaluate *in vivo* vitamin E status. While the metabolic end products for α-tocopherol (Schultz et al., 1995) and γ-tocopherol (Wechter et al., 1996) have been identified as α-CEHC and γ-CEHC (carboxy-ethyl-hydroxy-chroman) respectively, the specific cytochrome P450 isoforms utilized to initiate tocopherol metabolism remain subject to debate (Birringer et al., 2001; Sontag and Parker, 2002). Regardless, it appears that α-tocopherol and γ-tocopherol metabolism is initiated by ω-oxidation followed by stepwise β-oxidation until α-CEHC or γ-CEHC, respectively, is formed (Birringer et al., 2001). Reportedly, excretion of α-CEHC is minimal unless individuals are supplemented with α-tocopherol (Radosavac et al., 2002). Furthermore, α-tocopherol metabolism appears to be activated only after a plasma threshold of 30-40 µM α-tocopherol is achieved (Schuelke et al., 2000). In comparison to α-tocopherol, γ-tocopherol is more readily metabolized, resulting in lower plasma γ-tocopherol concentrations and urinary γ-CEHC concentrations that are >10-fold higher than urinary α-CEHC concentrations in unsupplemented individuals (Galli et al., 2002). In addition, α-tocopherol
supplementation also increases γ-tocopherol metabolism as demonstrated by higher urinary γ-CEHC and lower plasma γ-tocopherol concentrations (Morinobu et al., 2003). To date, no investigations have compared CEHCs between nonsmokers and cigarette smokers or determined if ascorbic acid supplementation increases plasma concentrations of tocopherols and therefore increases the production of CEHCs.

Oxidative stress is often an implicating factor for alterations in dietary antioxidant utilization. In fact, cigarette smokers have an increased metabolic turnover of ascorbic acid that has led to the dietary recommendation for these individuals to consume an additional 35 mg/day compared to nonsmokers (Institute of Medicine (U.S.). Panel on Dietary Antioxidants and Related Compounds., 2000). However, considerably less is known about the impact of oxidative stress on vitamin E utilization. In vitro investigations generally suggest that vitamin E is depleted with exposure to cigarette smoke (Eiserich et al., 1995; Handelman et al., 1996), but evidence from in vivo investigations is less conclusive. Some clinical investigations have indicated that cigarette smokers have lower plasma vitamin E concentrations than nonsmokers (Bolton-Smith et al., 1991; Mezzetti et al., 1995), whereas others have reported no apparent differences among these groups (Dietrich et al., 2003; Lykkesfeldt et al., 2000; Ross et al., 1995). In addition, kinetics studies that have supplemented smokers and nonsmokers with deuterium labeled α-tocopherols were also inconclusive. In one trial (Munro et al., 1997), smokers and nonsmokers were supplemented on a single occasion with deuterated α-tocopherols and then collected blood samples at 6, 12, and 27 h following the supplement. While smokers had lower plasma deuterated α-tocopherol concentrations at each of the time points studied, it could not be determined if these differences were due to differences in α-tocopherol absorption or due to its faster plasma clearance. In attempt to clarify these differences,
other investigators (Traber et al., 2001) conducted an additional trial. They supplemented smokers and nonsmokers with deuterated α-tocopherols for seven days and collected fasting blood samples on select days for three weeks in effort to evaluate differences in fractional disappearance rates. Although the smokers had a faster rate of deuterated α-tocopherol disappearance, this finding was not statistically significant because the investigation was likely underpowered.

Recent data from our laboratory (unpublished; Chapter 3 of this dissertation) using deuterium labeled α-tocopherols indicated that smokers, but not nonsmokers, had faster α-tocopherol disappearance that was correlated to plasma ascorbic acid concentrations. This observation was true despite similar average plasma ascorbic acid concentrations and dietary ascorbic acid intakes among the smokers and nonsmokers. However, the data suggested that if smokers had higher plasma ascorbic acid concentrations, then vitamin E disappearance would be normalized relative to nonsmokers.

Therefore, in this investigation we hypothesized that the higher magnitude of oxidative stress experienced by cigarette smokers compared with nonsmokers would lead to more rapid plasma depletion of deuterium-labeled tocopherols and to decreased plasma CEHC concentrations. In addition, we also hypothesized that ascorbic acid supplementation would alleviate oxidative stress and restore vitamin E disappearance kinetics. To test this hypothesis, we supplemented smokers and nonsmokers with ascorbic acid for two weeks and then orally administered deuterated α- and γ-tocopherols and measured their plasma tocopherols and CEHC disappearance kinetics using liquid chromatography/mass spectrometry.
4.3. MATERIALS AND METHODS

Subjects

This study protocol was approved by the Institutional Review Board for the protection of human subjects at Oregon State University. Participants were recruited by newspaper advertisements in the Corvallis, Oregon area. Twenty-two adults (12 nonsmokers; 10 smokers) who responded to our advertisements were enrolled because they met our study participation criteria: 18-35 years, healthy, non-nutritional supplement user > 6-months, < 7 h/week aerobic exercise, and were cigarette smokers or nonsmokers. Nonsmokers did not reside with anyone who smoked and smoking status was confirmed in all participants by radioimmunoassay of urinary cotinine (Diagnostic Products Corp, CA). A urinary cotinine concentration of >500 ng/mL was used as a cutoff to confirm smoking status as suggested by the manufacturer. Each subject signed an informed consent statement prior to enrollment in the study. To evaluate participants’ health, routine serum blood chemistry profiles, including lipid panels, were performed on all of the subjects at Good Samaritan Hospital (Corvallis, OR).

Materials

α-5,7-(CD₃)₂ tocopheryl acetate (d₆-α-tocopheryl acetate) and unlabeled α-tocopheryl acetate (d₀-α-tocopheryl acetate) were gifts from Dr. James Clark of Cognis Nutrition and Health. γ-3,4-(D) tocopheryl acetate (d₂-γ-tocopheryl acetate) was prepared from γ-tocopherol labeled with two deuterium atoms, as described (Lei
and Atkinson, 2001). The d₆-α- and d₂-γ-tocopheryl acetates were mixed in a 1:1 molar mixture, and capsules made of approximately 50 mg each. The d₆-α- to d₂-γ-tocopherol molar ratio was determined by liquid chromatography/mass spectrometry (LC/MS) to be 0.98. The internal standard, α-5,7,8-(CD₃)₃ tocopheryl acetate (d₉-α-tocopheryl acetate), was provided by Dr. Carolyn Good of The Bell Institute of Health and Nutrition, and was synthesized by Isotec, Inc. (Miamisburg, OH). For use as authentic standards 2,5,7,8-tetramethyl-2-(2′-carboxyethyl)-6-hydroxychroman (α-CEHC) and 2,7,8-trimethyl-2-(β-carboxyethyl)-6-hydroxychroman (γ-CEHC) (LLU-α) were gifts from W.J. Wechter of Loma Linda University.

HPLC-grade methanol, hexane, ethanol, and glacial acetic acid were obtained from Fisher (Fair Lawn, NJ). Unlabeled γ-tocopherol (d₀-γ-tocopherol), trolox, ascorbic acid, potassium hydroxide (KOH), butylhydroxy toluene (BHT), lithium perchlorate, trolox, and β-glucuronidase (type H-1, contains minimum 300,000 U/g β-glucuronidase activity and minimum 10,000 U/g sulfatase activity) were from Sigma-Aldrich (St. Louis, MO). Diethyl ether was obtained from Mallinckrodt Baker, Inc. (Phillipsburg, NJ).

**Study Design**

This investigation was conducted as a double-blind, placebo-controlled randomized-cross over study. Participants were randomized to 17-day treatments of either ascorbic acid (500 mg; twice daily) or placebo (70.5% calcium phosphate, dibasic; 29.5% cellulose; 0.5% magnesium stearate), which were kindly provided by Dr. Steven Yannicelli of Pharmavite, LLC (Northridge, CA). Participants were instructed to ingest one supplement in the morning and one in the evening. On days when blood samples were obtained, participants were instructed to take their
“morning” supplement after the blood draw and their “evening” supplement at least 12 hours prior to arriving at the study center.

On day 14, participants returned to the study center in the morning and were provided with a standard breakfast (560 kcal with \(^{20}\%\) of the calories from fat) followed by oral administration of the mixed tocopherol capsule containing an equal molar mixture of \(~50\text{ mg each of } \text{d}_6\)-\(\alpha\)- and \(\text{d}_2\)-\(\gamma\)-tocopheryl acetates. Blood samples were collected at baseline (0 h) and then 3, 6, 9, 12, 24, 36, 48 and 72 h after the breakfast and oral administration of the deuterated tocopherol capsule. Lastly, participants were instructed to follow a low ascorbic acid diet during each 17-day supplementation period. After a 3-month wash-out period, participants returned to the study center to receive the alternate treatment and completed the study as just described.

Blood samples were collected from the antecubital vein into evacuated tubes containing 0.05 mL 15\% (wt:vol) EDTA, sodium heparin, or no additives (Becton Dickinson, Franklin Lakes, NJ). Plasma was promptly separated by centrifugation at 4\(^\circ\)C for 15 min at 500 x \(g\) (model TJ-6; Beckman Coulter, Palo Alto, CA) and stored at -80\(^\circ\)C until analyzed. Serum was obtained from tubes without additives that were allowed to clot for 2-hours prior to separation by centrifugation as described above. After heparinized plasma was separated, an aliquot was acidified (1:1) with 10\% PCA (perchloric acid) containing 1 mM diethylenetriaminepentaacetic acid (DTPA). This sample was then centrifuged (5 min, 15,000 x \(g\), 4\(^\circ\)C; Eppendorf Centrifuge 5415R, Hamburg, Germany), the supernatant was removed, snap frozen, and stored at -80\(^\circ\)C for future analysis of ascorbic and uric acid.
Measurement of plasma antioxidants and lipids

Labeled and unlabeled plasma α- and γ-tocopherols were extracted (Podda et al., 1996a) and measured by LC/MS using negative atmospheric pressure chemical ionization (-APCI) as previously described (Leonard et al., 2003; Vaule et al., 2004). Plasma ascorbic acid and uric acid were measured by HPLC with amperometric detection as previously described (Frei et al., 1989b). Plasma triglycerides and total cholesterol were determined by standard clinical assays (Sigma, St. Louis, MO). Plasma free F₂α-isoprostanes were measured by a previously described method (Morrow and Roberts, 1994).

Measurement of plasma CEHCs

Plasma CEHCs were extracted using a modified method (Lodge et al., 2000). Briefly, internal standard (trolox) was added to 0.5 mL of serum, as was 10 µL ascorbic acid (10 g/L) to stabilize CEHCs. The CEHC conjugates were then hydrolyzed by the addition of 100 µL enzyme solution (1 mg β-glucuronidase in 100 µL 10 mM potassium phosphate buffer, pH 6.8). Following 30 min incubation at 37º C, the samples were acidified by the addition of 10 µL 12 M HCL. CEHCs were subsequently extracted with diethyl ether, mixing by hand inversion. An aliquot of the ether fraction was collected, dried under N₂ and the residue resuspended in 1:1 (vol:vol) H₂O:MeOH, containing 0.05% (vol:vol) acetic acid and 0.05% (wt:vol) ascorbic acid.

Extracted CEHCs were analyzed by LC/MS using a Waters 2690 Separations Module (Milford, MA). Instrument control and acquisition were done using the Waters Masslynx V. 3.4 software. The column used was a SymmetryShield™ RP-18 column (3.0 cm x 150 mm, 3.5 µm particle, Waters, Milford, MA) with a SymmetryShield™ Sentry™ RP-18 precolumn (3.9 cm x 20 mm, 3.5 µm particle,
Waters, Milford, MA). For the solvent delivery, a modified gradient method was used (Himmelfarb et al., 2003). The system was first equilibrated with 50% methanol (containing 0.05% acetic acid) for 1 min, followed by a linear gradient to 80% methanol (containing 0.05% acetic acid) in 6 min at a flow rate of 0.20 mL/min. These conditions were maintained for 15 min, followed by a 5 min wash period with 95% methanol (containing 0.05% acetic acid), at which time original conditions were established and run for 5 min prior to injection of the succeeding sample. The other mobile phase used for the gradient was H2O, also containing 0.05% acetic acid.

Samples were analyzed using a Micromass (Manchester, England) ZQ 2000 single-quadrupole mass spectrometer with an electrospray ionization source (ESI). The capillary voltage was set to 2.5 kV, and the sample cone voltage at -30 V. The desolvation temperature was set at 150°C. The desolvation gas (nitrogen) was set to 160 L/hour, the nebulizer gas (nitrogen) at 80 psi., and the cone gas (nitrogen) at 50 L/hour. Single ion recording (SIR) mass-to-charge (m/z) ratio data were obtained for \(d_0-\alpha\)-CEHC, \(m/z\) 277.8; \(d_0-\alpha\)-CEHC, \(m/z\) 283.8; \(d_0-\gamma\)-CEHC, \(m/z\) 263.8; \(d_2-\gamma\)-CEHC, \(m/z\) 265.8; and trolox, \(m/z\) 249.8. The dwell time for each of the ions was set at 0.20 second. Typical retention times were 14.2, 14.6, and 15.4 min for trolox, \(\gamma\)-CEHC’s, and \(\alpha\)-CEHC’s, respectively. At this time deuterium labeled CEHC standards are not commercially available, and thus quantitation was performed using an internal standard of trolox. Sample CEHC concentrations were calculated from the peak area of the corresponding ion to that of the trolox peak. The working linear range for quantitation was 0.2 to 20 pmol injected and the low limit of detection was 0.08 pmol injected.
**Evaluation of Plasma Tocopherols and Metabolite Disappearance Kinetics**

The maximum tocopherol and CEHC concentrations (C_{max}) and the time of maximum concentration (T_{max}) were determined by visual inspection of the data. Plasma exponential disappearance rates of d_6-\alpha-tocopherol, d_2-\gamma-tocopherol and d_2-\gamma-CEHC were estimated by linear regression on a natural log scale, for each subject and each treatment, by fitting a line from C_{max} through the remaining blood sampling time points. Half-lives of these compounds were directly calculated as t_{1/2} = \ln(2)/\text{exponential disappearance rate constant.}

The breakfast generated little within subject variation in the plasma cholesterol and triglyceride concentrations. Although lipid standardized (cholesterol plus triglycerides) unlabeled and labeled tocopherol concentrations were calculated and statistical analysis performed, similar findings were obtained whether or not the data were adjusted for plasma lipid concentrations. Therefore, only unadjusted unlabeled and labeled tocopherol concentrations are discussed.

**Statistical Analysis**

All data are expressed as means ± SE unless otherwise noted. Statistical analysis was conducted using GraphPad Prism (Version 4.0) software (GraphPad Software, San Diego, CA). Data were analyzed by either the student’s t-test for baseline comparisons between smokers and nonsmokers or by using two-way ANOVA with repeated measures followed by Bonferroni’s post hoc analysis when appropriate. Results were considered to be statistically significant at an \alpha-level of \( p < 0.05 \).
4.4. RESULTS

Participant Characteristics

Participant characteristics prior to study randomization are shown in Table 4.1. Smokers (n = 6 males, 4 females) and nonsmokers (n = 6 males, 6 females) did not differ with respect to age, height, weight, BMI, or plasma uric acid. However, nonsmokers had ~27% higher plasma ascorbic acid concentrations than smokers (63.9 ± 4.0 vs. 50.4 ± 5.3 µM), respectively. Urinary cotinine was measured during each supplementation period. In participants who identified themselves as smokers, urinary cotinine concentrations were > 500 ng/mL, but these concentrations did not correlate with self-reported cigarettes/day (p = 0.331). All subjects had hematological values within normal limits (Table 4.2) and values for all parameters were similar between smokers and nonsmokers.

Ascorbic Acid Treatment

Among the nonsmokers only, there was a 28% decrease (p < 0.01) in plasma ascorbic acid from pre-randomization to the placebo trial, which was attributed to good compliance in following a low ascorbic acid diet. Ascorbic acid supplementation resulted in approximately a two-fold increase (p < 0.0001) in plasma ascorbic acid concentrations compared to placebo treatment regardless of participation group. Smokers’ and nonsmokers’ plasma ascorbic acid concentrations increased from 42.7 ± 5.7 µM to 96.8 ± 2.9 µM and 45.9 ± 3.8 µM to 89.9 ± 3.7 µM, respectively (Figure 4.1). In addition, plasma ascorbic acid concentrations were
similar between participation groups during the placebo trial and the ascorbic acid supplementation trials.

**Disappearance Rates and Half-Lives of Labeled Tocopherols and γ-CEHC**

Plasma $d_6$-$\alpha$-tocopherol, $d_2$-$\gamma$-tocopherol, and $d_2$-$\gamma$-CEHC concentrations for nonsmokers and smokers are shown in Figure 4.2. Neither, ascorbic acid supplementation ($p > 0.05$) or smoking ($p > 0.05$) had any significant effect on $C_{\text{max}}$ for $d_6$-$\alpha$-tocopherol, $d_2$-$\gamma$-tocopherol, or $d_2$-$\gamma$-CEHC concentrations. Similarly, no treatment ($p > 0.05$) or group ($p > 0.05$) effects were observed with regard to $T_{\text{max}}$ of $d_6$-$\alpha$-tocopherol or $d_2$-$\gamma$-tocopherol. However, smoking ($p = 0.0428$), but not ascorbic acid supplementation ($p > 0.05$), resulted in maximal $d_2$-$\gamma$-CEHC concentrations that peaked ~5 hours earlier among the smokers (12.0 ± 1.0 h) compared to the nonsmokers (17.0 ± 2.1 h).

Both smoking (main effect $p = 0.0138$) and ascorbic acid supplementation (main effect $p = 0.0383$) had significant effects on $d_6$-$\alpha$-tocopherol exponential disappearance rates (Figure 4.3A). During the placebo trial, $d_6$-$\alpha$-tocopherol exponential disappearance rates were ~41% faster among smokers than nonsmokers ($p < 0.05$) and treatment with ascorbic acid significantly lowered the smokers’ $d_6$-$\alpha$-tocopherol exponential disappearance rates by ~24% ($p < 0.05$), but no changes were observed for the nonsmokers ($p > 0.05$). Half-lives for smokers’ $d_6$-$\alpha$-tocopherol during the placebo and ascorbic acid trials were 27.1 ± 2.5 and 35.4 ± 3.8 hours, respectively. For nonsmokers, $d_6$-$\alpha$-tocopherol half-lives were 36.5 ± 2.0 and 41.4 ± 5.6 hours during the placebo and ascorbic acid trials, respectively. Therefore, smokers had faster $d_6$-$\alpha$-tocopherol disappearance than nonsmokers that was corrected by ascorbic acid supplementation.
Ascorbic acid also significantly reduced (main effect \( p = 0.0462 \)) exponential disappearance rates of d\(_2\)-\(\gamma\)-tocopherol (Figure 4.3B) but smoking had no effect (main effect \( p = 0.1008 \)) despite disappearance rates among the smokers that were \( \sim 34\% \) higher than nonsmokers during the placebo trial. By post-hoc analysis, ascorbic acid supplementation affected the smokers only and reduced their d\(_2\)-\(\gamma\)-tocopherol disappearance rates by 30% \( (p < 0.05) \). Corresponding half-lives of d\(_2\)-\(\gamma\)-tocopherol for the smokers during the placebo and ascorbic acid trials were 11.2 ± 1.5 and 15.7 ± 2.0 hours, respectively. Among the nonsmokers, these half-lives were 13.7 ± 0.9 and 14.5 ± 1.1 hours during the placebo and ascorbic acid supplementation periods, respectively. Therefore, smokers were able to correct faster d\(_2\)-\(\gamma\)-tocopherol disappearance with ascorbic acid supplementation.

Increased vitamin E metabolism might provide an explanation for faster vitamin E disappearance. However, the data demonstrate that neither smoking (main effect \( p = 0.795 \)) or ascorbic acid supplementation (main effect \( p = 0.764 \)) affected d\(_2\)-\(\gamma\)-CEHC exponential disappearance rates (Figure 4.3C). Furthermore, d\(_6\)-\(\alpha\)-CEHC was not detectable during the analysis which further supports that vitamin E metabolism was not increased by smoking or ascorbic acid supplementation. Calculated half-lives of d\(_2\)-\(\gamma\)-CEHC for the nonsmokers during the placebo and ascorbic acid supplementation periods were 15.6 ± 1.2 and 17.8 ± 1.1 hours, respectively. For the smokers, the d\(_2\)-\(\gamma\)-CEHC half-lives were 19.2 ± 1.5 and 16.2 ± 1.7 during the placebo and ascorbic acid supplementation periods, respectively.

It should also be noted that the nonsmokers’ d\(_2\)-\(\gamma\)-tocopherol and d\(_2\)-\(\gamma\)-CEHC disappearance rates were similar (Figure 4.4). However, the smokers’ d\(_2\)-\(\gamma\)-tocopherol disappearance rates compared to their d\(_2\)-\(\gamma\)-CEHC rates were \( \sim 43\% \) higher during the placebo trial compared to the ascorbic acid trial \( (p < 0.01) \). This observation suggests
that the degree of oxidation of d₂-γ-tocopherol is far greater than its metabolism and that ascorbic acid supplementation effectively reduces d₂-γ-tocopherol oxidation.

Lastly, overall comparison of exponential disappearance rates between d₆-α-tocopherol and d₂-γ-tocopherol demonstrated that plasma d₂-γ-tocopherol disappeared ~3 times faster than d₆-α-tocopherol regardless of smoking (p > 0.05) or ascorbic acid supplementation (p > 0.05). Based on the similarity of d₂-γ-tocopherol and d₂-γ-CEHC disappearance rates, it is most likely that the accelerated disappearance of d₂-γ-tocopherol compared to d₆-α-tocopherol is due to its greater metabolism. Unfortunately, no direct comparison between exponential disappearance rates of d₂-γ-CEHC and d₆-α-CEHC could be calculated because d₆-α-CEHC concentrations were below the detection limits throughout the LC/MS analysis. However, the lack of d₆-α-CEHC detection further confirms that γ-tocopherol is more readily metabolized in comparison to α-tocopherol and that α-tocopherol disappearance most likely occurred through oxidation and not through increased metabolism.

**Labeled Tocopherol Concentrations at 72 hours**

In smokers, exponential disappearance rates for d₆-α-tocopherol and d₂-γ-tocopherol were significantly lowered by ascorbic acid supplementation. Therefore, labeled tocopherol concentrations at the end of the investigation (72 hrs) would be expected to be higher during the ascorbic acid trial compared to the placebo trial. In fact, smokers’ plasma d₆-α-tocopherol concentrations at 72 hrs were 40% higher (p = 0.0024) with ascorbic acid supplementation (0.66 ± 0.12 µM) compared to the placebo supplementation period concentrations (0.47 ± 0.14 µM).

A similar analysis was performed for d₂-γ-tocopherol. However, due to the rapid disappearance kinetics observed for d₂-γ-tocopherol, particularly during the placebo period, the distributions of d₂-γ-tocopherol were not normally distributed and
therefore, the Mann Whitney test was used for this particular analysis. Smokers had significantly higher ($p = 0.0315$) plasma $d_2$-$\gamma$-tocopherol concentrations during the ascorbic acid period (median = 0.051 µM; mean ± SE = 0.074 ± 0.018 µM) compared to the placebo period (median = 0.0009 µM; mean ± SE = 0.040 ± 0.019 µM) which supports the observation that smokers’ $d_2$-$\gamma$-tocopherol disappearance rate kinetics were reduced by 30% with ascorbic acid supplementation.

Unlabeled Plasma Tocopherols and $\gamma$-CEHC

Prior to taking the deuterated vitamin E capsule, but after 2-weeks supplementation with ascorbic acid ($t = 0$ h), smokers and nonsmokers did not differ with respect to unlabeled plasma ($d_0$-) $\alpha$-tocopherol, $\gamma$-tocopherol, $\alpha$-CEHC or $\gamma$-CEHC (Figure 4.5). It should also be noted that most participants had little (< 5 nM) or undetectable plasma $d_0$-$\alpha$-CEHC. However, on average, plasma $d_0$-$\alpha$-CEHC concentrations were 17-fold lower than $d_0$-$\gamma$-CEHC concentrations while, $d_0$-$\alpha$-tocopherol concentrations were ~9-fold higher than $d_0$-$\gamma$-tocopherol concentrations.

Plasma F$_{2\alpha}$-Isoprostanes

Plasma F$_{2\alpha}$-isoprostanes were measured to assess the magnitude of oxidative stress experienced by the smokers. On average, smokers had ~35% higher F$_{2\alpha}$-isoprostanes compared to the nonsmokers. However, smokers’ plasma F$_{2\alpha}$-isoprostanes concentrations were not significantly higher than those of the nonsmokers during the ascorbic acid ($p > 0.05$) or placebo ($p > 0.05$) supplementation periods (Figure 4.6). In addition, ascorbic acid supplementation had no impact on reducing F$_{2\alpha}$-isoprostanes concentrations ($p = 0.930$) and no correlation was observed between plasma ascorbic acid and plasma F$_{2\alpha}$-isoprostanes ($p = 0.449$).
4.5. DISCUSSION

Short-term intervention with ascorbic acid supplementation can effectively reduce plasma $\alpha$-tocopherol and $\gamma$-tocopherol disappearance rates in cigarette smokers. In this investigation, we expected that smokers would have greater plasma tocopherol disappearance rates and that ascorbic acid supplementation would attenuate oxidative stress and thus restore $\alpha$-tocopherol and $\gamma$-tocopherol disappearance. On the contrary, ascorbic acid supplementation did not decrease lipid peroxidation ($F_{2\alpha}$-isoprostanes) or increase tocopherol metabolism. Therefore, the mechanism for restored plasma tocopherol kinetics and plasma tocopherol concentrations must be due to the ability of ascorbic acid to directly reduce $\alpha$- and $\gamma$-tocopheroxyl radicals to their nonoxidized forms.

In this investigation, smokers and nonsmokers were provided a 1:1 molar mixture of ~50 mg each of $d_6$-$\alpha$-tocopherol acetate and $d_2$-$\gamma$-tocopherol acetate that was preceded by a 2-week intervention of ascorbic acid (500 mg; twice daily) or placebo. Ascorbic acid supplementation effectively ~doubled plasma ascorbic acid concentrations (Figure 4.1) in both smokers and nonsmokers but only reduced smokers’ plasma tocopherol disappearance kinetics (Figure 4.3). The favorable reductions in $d_6$-$\alpha$-tocopherol and $d_2$-$\gamma$-tocopherol disappearance rates among the smokers were attributed to higher plasma ascorbic acid concentrations and not due to any significant changes in $C_{\text{max}}$ or $T_{\text{max}}$ (Figure 4.2) of these deuterated tocopherols or to any changes in unlabeled plasma $\alpha$- and $\gamma$-tocopherol concentrations (Figure 4.5). In addition, the smokers’ greater $d_6$-$\alpha$-tocopherol and $d_2$-$\gamma$-tocopherol disappearance...
rates observed during the placebo trial were not attributed to increased metabolism of tocopherols to CEHCs as demonstrated by the absence of detectable d_{6}-\alpha-CEHC and the unchanged disappearance rates for d_{2}-\gamma-CEHC (Figure 4.3). The lack of increased vitamin E metabolism occurring in smokers is also supported by the ratio of disappearance rates between d_{2}-\gamma-tocopherol and d_{2}-\gamma-CEHC (Figure 4.4). This calculation demonstrates that ascorbic acid supplementation had a significant effect in favorably reducing disappearance rates of \gamma-tocopherol in smokers while metabolism remained unchanged.

With regard to d_{2}-\gamma-tocopherol, it was not possible to discern from the available data if the reduced rates of plasma disappearance were attributed to less nitration (Leonard et al., 2003) or less oxidation of \gamma-tocopherol. However, the reduction of plasma disappearance rates of d_{6}-\alpha-tocopherol were attributed to less oxidation since \alpha-tocopherol does not have an unsubstituted position on the chromanol head that can trap reactive nitrogen oxide species as does \gamma-tocopherol (Christen et al., 1997). Lastly, it should be noted that d_{2}-\gamma-tocopherol disappeared from the plasma nearly 3-times faster than d_{6}-\alpha-tocopherol, regardless of group or treatment. Our ability to detect d_{2}-\gamma-CEHC and not d_{6}-\alpha-CEHC also suggests that d_{2}-\gamma-tocopherol is more rapidly metabolized, a finding that is also supported by the ~3-fold lower half-lives for d_{2}-\gamma-tocopherol compared to d_{6}-\alpha-tocopherol.

Among the smokers only, ascorbic acid supplementation effectively retarded \alpha-tocopherol and \gamma-tocopherol disappearance kinetics by 24% and 30%, respectively, which suggests that ascorbic acid and plasma tocopherols directly interact in vivo only when challenged by an oxidative stress, such as cigarette smoke. Our results directly relate to the substantial health benefits observed in the ASAP (Antioxidant Supplementation in Atherosclerosis Prevention) trial (Salonen et al., 2000; Salonen et
al., 2003) in which co-supplementation with vitamin C (250 mg) and vitamin E (91 mg \( \alpha \)-tocopherol) for three years significantly reduced carotid artery intima-media thickness among hypercholesterolemic male smokers (45-69 years of age) who were diagnosed prior to study enrollment with common carotid atherosclerosis whereas individual nutrient supplementation offered no health benefit.

The results from our \textit{in vivo} investigation are further supported by various \textit{in vitro} studies conducted under induced conditions of oxidative stress. Such studies have demonstrated that ascorbic acid either spares \( \alpha \)-tocopherol from oxidation (Frei et al., 1989a; Huang and May, 2003) or it regenerates a reduced \( \alpha \)-tocopherol from an \( \alpha \)-tocopheroxyl radical. Since ascorbic acid is water-soluble and tocopherols are lipid-soluble, it is likely that these nutrients interact at the interface of these two compartments once \( \alpha \)- or \( \gamma \)-tocopherol becomes oxidized (Buettner, 1993). Due to the similar structures of \( \alpha \)- and \( \gamma \)-tocopherol as well as the ability of both of these nutrients to become oxidized and prevent lipid peroxidation (Jiang and Ames, 2003; Willcox et al., 2003), it is plausible that ascorbic acid interacts with these tocopherols in a similar manner. Alternatively, ascorbic acid might be preventing peroxynitrite interactions (Kirsch and de Groot, 2000) with \( \gamma \)-tocopherol that could lead to the formation of nitro-\( \gamma \)-tocopherol, or it could be reducing lipid peroxidation (Polidori et al., 2004). However, from our investigation we observed no decreases in F\( \alpha \)-isoprostanes due to ascorbic acid supplementation. Therefore, lower disappearance rates of \( \alpha \)- and \( \gamma \)-tocopherol observed during the ascorbic acid trial must be due to ability of ascorbic acid to reduce (i.e. recycle) an oxidized tocopherol moiety.

It was expected that plasma F\( \alpha \)-isoprostanes would provided a measure of the degree of oxidative stress experienced by the smokers. Previous studies demonstrated that smokers had higher plasma F\( \alpha \)-isoprostanes concentrations than nonsmokers
(Morrow et al., 1995) and that supplementation with ascorbic acid (500 mg/d) for 2-months significantly reduced plasma F2α-isoprostanes (Dietrich et al., 2002). In our study, smokers had ~35% higher F2α-isoprostanes compared to nonsmokers during both study periods. However, due to the high variability of this marker among this cohort of smokers no significant differences were observed between smokers and nonsmokers, suggesting the possibility that our sample size was too small and that perhaps our intervention was too short in duration to significantly reduce plasma lipid peroxidation. Therefore, we can only speculate from these data that a smoker cohort with a higher degree of lipid peroxidation would have even faster tocopherol disappearance rates since it is reported that the reaction of α-tocopherol with peroxyl radicals is ~1000 times more favorable than the reaction of peroxyl radicals with polyunsaturated fatty acids (Buettner, 1993; Burton et al., 1983).

The results of this investigation suggest that smokers have higher dietary requirements of vitamin E than nonsmokers. However, the cohort of smokers in this investigation were young (19-24 years), with a limited history (< 5 years) and frequency of smoking (6-16 cigarettes/d), and plasma ascorbic acid concentrations at baseline that were within the normal physiological range (Institute of Medicine (U.S.), Panel on Dietary Antioxidants and Related Compounds., 2000). Therefore, we can only speculate that older smokers with a longer history, greater frequency, and lower ascorbic acid concentrations would have even faster vitamin E disappearance kinetics because aging itself is a well known source of oxidative stress (Ames et al., 1993).

In conclusion, our data support that oxidative stress increased α-tocopherol and γ-tocopherol disappearance rates and that short-term ascorbic acid supplementation attenuated these tocopherols’ disappearance rates. Consequently, cigarette smokers require higher dietary intakes of ascorbic acid and vitamin E to maintain similar
plasma concentrations of vitamin E as nonsmokers. Recent dietary data (Maras et al., 2004) suggested that only 8% and 2.4% of men and women, respectively, in the United States consume diets that meet the Estimated Average Requirement for α-tocopherol. In light of these dietary data and the data from this investigation, it would seem necessary for smokers to strive to consume a diet that contains at least the RDAs (Institute of Medicine (U.S.). Panel on Dietary Antioxidants and Related Compounds., 2000) for vitamin E (15 mg/d α-tocopherol) and vitamin C (125 and 110 mg/d for male and female smokers, respectively). In addition, further trials are warranted to determine if other dietary antioxidants can attenuate α- and γ-tocopherol disappearance.
4.6. ACKNOWLEDGMENTS

We are especially grateful to the study participants for their cooperation during this investigation. The authors would also like to thank the Natural Source Vitamin E Association for partial support of the purchase of the LC/MS. Special thanks to Wendy McMahan of the Montine lab for providing the F$_{2\alpha}$-isoprostane analysis and to the National Institutes of Health for providing the financial support to MGT (NIH DK 59576) and TJM (AG05144 and AG16835), which made this study possible.
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Nonsmokers (n = 12)</th>
<th>Smokers (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>23.1 ± 2.5</td>
<td>21.1 ± 1.7</td>
</tr>
<tr>
<td>Height (m)</td>
<td>1.73 ± 0.09</td>
<td>1.73 ± 0.09</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>68.2 ± 3.6</td>
<td>68.2 ± 3.5</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>24.6 ± 2.8</td>
<td>23.2 ± 3.9</td>
</tr>
<tr>
<td>Nutrition supplements</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Ascorbic Acid (µM)</td>
<td>63.9 ± 14.9</td>
<td>50.4 ± 18.5</td>
</tr>
<tr>
<td>Uric Acid (µM)</td>
<td>283 ± 87</td>
<td>310 ± 78</td>
</tr>
<tr>
<td>Cigarettes/day</td>
<td>0</td>
<td>10.6 ± 3.7</td>
</tr>
<tr>
<td>Urinary cotinine (ng/mL)</td>
<td>27 ± 13</td>
<td>2587 ± 1615</td>
</tr>
</tbody>
</table>

Table 4.1. Baseline participant characteristics
Baseline data are shown as mean ± SD. *a* At screening, ascorbic acid was 21% lower among the smokers compared to nonsmokers (p = 0.040). *b* Urinary cotinine are mean values from both trials since no effect due to ascorbic acid was observed. Urinary cotinine did not correlate with self-reported cigarettes/day (p = 0.331).
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Normal Range</th>
<th>Nonsmokers</th>
<th>Smokers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>13.0 - 16.8</td>
<td>15.9 ± 1.3</td>
<td>16.3 ± 1.1</td>
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<tr>
<td>Hematocrit (%)</td>
<td>38 – 49</td>
<td>46 ± 3</td>
<td>47 ± 3</td>
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<tr>
<td>Cholesterol (mmol/L)</td>
<td>&lt; 5.16</td>
<td>4.32 ± 0.69</td>
<td>4.05 ± 0.48</td>
</tr>
<tr>
<td>HDL (mmol/L)</td>
<td>&gt; 1.03</td>
<td>1.57 ± 0.51</td>
<td>1.50 ± 0.43</td>
</tr>
<tr>
<td>LDL (mmol/L)</td>
<td>&lt; 3.36</td>
<td>2.35 ± 0.71</td>
<td>2.05 ± 0.57</td>
</tr>
<tr>
<td>VLDL (mmol/L)</td>
<td>&lt; 1.03</td>
<td>0.40 ± 0.17</td>
<td>0.50 ± 0.20</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>&lt; 2.26</td>
<td>0.88 ± 0.38</td>
<td>1.09 ± 0.44</td>
</tr>
<tr>
<td>Sodium (mEq/L)</td>
<td>135 – 145</td>
<td>138 ± 2</td>
<td>140 ± 2</td>
</tr>
<tr>
<td>Potassium (mEq/L)</td>
<td>3.5 - 5.1</td>
<td>4.5 ± 0.5</td>
<td>4.5 ± 0.6</td>
</tr>
<tr>
<td>Chloride (mEq/L)</td>
<td>100 – 111</td>
<td>101 ± 2</td>
<td>102 ± 2</td>
</tr>
<tr>
<td>Bicarbonate (mEq/L)</td>
<td>22 – 30</td>
<td>30 ± 3</td>
<td>30 ± 2</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>70 – 105</td>
<td>80 ± 6</td>
<td>81 ± 8</td>
</tr>
<tr>
<td>BUN (mg/dL)</td>
<td>6 – 19</td>
<td>13 ± 3</td>
<td>13 ± 3</td>
</tr>
<tr>
<td>Creatinine (mg/dL)</td>
<td>0.4 - 1.1</td>
<td>0.8 ± 0.2</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>BUN/creatinine ratio</td>
<td>6 – 30</td>
<td>16 ± 2</td>
<td>15 ± 3</td>
</tr>
<tr>
<td>Calcium (mg/dL)</td>
<td>8.4 - 10.2</td>
<td>9.8 ± 0.3</td>
<td>10.0 ± 0.4</td>
</tr>
<tr>
<td>Phosphorus (mg/dL)</td>
<td>2.7 - 4.5</td>
<td>3.6 ± 0.6</td>
<td>3.9 ± 0.7</td>
</tr>
<tr>
<td>Total protein (g/dL)</td>
<td>6.4 - 8.3</td>
<td>7.1 ± 0.3</td>
<td>7.2 ± 0.3</td>
</tr>
<tr>
<td>Albumin (g/dL)</td>
<td>3.4 - 5.0</td>
<td>4.6 ± 0.3</td>
<td>4.7 ± 0.3</td>
</tr>
<tr>
<td>SGOT (U/L)</td>
<td>0 – 31</td>
<td>23 ± 5</td>
<td>21 ± 5</td>
</tr>
<tr>
<td>LDH (U/L)</td>
<td>94 – 250</td>
<td>183 ± 19</td>
<td>174 ± 29</td>
</tr>
<tr>
<td>SGPT (U/L)</td>
<td>0 – 31</td>
<td>21 ± 9</td>
<td>18 ± 4</td>
</tr>
<tr>
<td>Alkaline phosphatase (U/L)</td>
<td>39 – 117</td>
<td>59 ± 21</td>
<td>64 ± 16</td>
</tr>
<tr>
<td>Gamma GT (U/L)</td>
<td>7 – 33</td>
<td>18 ± 8</td>
<td>18 ± 4</td>
</tr>
<tr>
<td>Total bilirubin (mg/dL)</td>
<td>0.0 - 1.0</td>
<td>0.8 ± 0.3</td>
<td>0.7 ± 0.2</td>
</tr>
<tr>
<td>Globulin (g/dL)</td>
<td>2.3 - 3.5</td>
<td>2.5 ± 0.3</td>
<td>2.5 ± 0.3</td>
</tr>
</tbody>
</table>

Table 4.2. Subjects' blood chemistry and hematological values at screening
All data are shown as mean ± SD. Nonsmokers and smokers had similar blood chemistries and were within the normal reference range (data from Good Samaritan Hospital).
Ascorbic acid did not differ between nonsmokers and smokers during either of the treatments. However, nonsmokers had a 28% decline in plasma ascorbic acid during the placebo supplementation period compared to baseline (data not shown; $p < 0.01$). Ascorbic acid supplementation resulted in ~2-fold increase in plasma ascorbic acid in both the nonsmokers and smokers compared to the placebo supplementation period ($p < 0.0001$).

**Figure 4.1. Plasma ascorbic acid concentrations following placebo and ascorbic acid supplementation**

Ascorbic acid did not differ between nonsmokers and smokers during either of the treatments. However, nonsmokers had a 28% decline in plasma ascorbic acid during the placebo supplementation period compared to baseline (data not shown; $p < 0.01$). Ascorbic acid supplementation resulted in ~2-fold increase in plasma ascorbic acid in both the nonsmokers and smokers compared to the placebo supplementation period ($p < 0.0001$).
Figure 4.2. Plasma concentrations of \(d_6\)-\(\alpha\)-tocopherol, \(d_2\)-\(\gamma\)-tocopherol and \(d_2\)-\(\gamma\)-CEHC of nonsmokers and smokers during placebo and ascorbic acid trials

Smokers and nonsmokers responded similarly with respect to \(C_{\text{max}}\) for \(d_6\)-\(\alpha\)-tocopherol, \(d_2\)-\(\gamma\)-tocopherol or \(d_2\)-\(\gamma\)-CEHC and no effect of ascorbic acid supplementation was observed. In addition, a similar effect was observed for \(T_{\text{max}}\) of \(d_6\)-\(\alpha\)-tocopherol and \(d_2\)-\(\gamma\)-tocopherol between groups and treatments, but \(d_2\)-\(\gamma\)-CEHC peaked \(~5\) h earlier among the smokers \((p = 0.0428)\) regardless of treatment.
Figure 4.3. Plasma exponential disappearance rates of d₆-α-tocopherol, d₂-γ-tocopherol and d₂-γ-CEHC

Among the smokers only, ascorbic acid supplementation reduced d₆-α-tocopherol (A) and d₂-γ-tocopherol (B) exponential disappearance rates by 24% \((p < 0.05)\) and 30\% \((p < 0.05)\), respectively. No effect was observed for d₂-γ-CEHC (C) exponential disappearance rates for either group with ascorbic acid supplementation.
Figure 4.4. Comparison of exponential disappearance rates between d$_{2}$-$\gamma$-tocopherol and d$_{2}$-$\gamma$-CEHC.

As shown, d$_{2}$-$\gamma$-tocopherol and d$_{2}$-$\gamma$-CEHC disappearance rates were similar ($p < 0.05$) among nonsmokers regardless of treatment. However, smokers' d$_{2}$-$\gamma$-tocopherol disappearance rates compared to their d$_{2}$-$\gamma$-CEHC rates were $\sim$43% higher during the placebo trial compared to the ascorbic acid trial ($p < 0.05$) which suggests that $\gamma$-tocopherol oxidation is much greater than its metabolism.
Two-weeks of ascorbic acid supplementation did not significantly change baseline (t = 0 h) plasma d0-α-tocopherol (A), d0-γ-tocopherol (B), d0-α-CEHC (C), or d0-γ-CEHC concentrations in the nonsmokers or smokers. Plasma d0-α-tocopherol concentrations were ~9-fold higher than d0-γ-tocopherol, but plasma d0-α-CEHC concentrations were ~17-fold lower than d0-γ-CEHC.
Despite ~35% higher F$_{2\alpha}$-isoprostanes among the smokers in each trial, these differences were not statistically different from the nonsmokers.
CHAPTER 5

DISCUSSION
The investigations presented in this dissertation demonstrate that cigarette smoking increases vitamin E utilization in humans. For years, it has been speculated that the enormous radical burden caused directly by cigarette smoke and indirectly from its associated inflammatory response should alter vitamin E utilization. However, prior to our investigations, no clear or convincing evidence from human trials have demonstrated such a relationship.

Using cigarette smokers as a free-living model of oxidative stress, we tested the hypothesis that oxidative and nitrative stress increases vitamin E utilization. In Chapter 2, we explored the hypothesis that the increased nitrosative stress of cigarette smoking increases nitration of γ-tocopherol. In Chapter 3, smokers and nonsmokers achieved a similar level of deuterated vitamin E status, then we tested whether vitamin E disappearance was similar in the two groups. In Chapter 4, the role of oxidative stress on vitamin E disappearance in smokers was evaluated by supplementing smokers and nonsmokers with vitamin C or placebo for 2 weeks in a cross-over design, then measuring vitamin E kinetics. These investigations demonstrated that cigarette smoking is a causative factor for increased nitration of plasma γ-tocopherol, higher rates of plasma α-tocopherol disappearance, and lower urinary excretion of the α-tocopherol metabolite (α-CEHC) (Figure 5.1). In addition, plasma vitamin E disappearance kinetics among smokers, but not nonsmokers, were associated with plasma ascorbic acid concentrations. From the acute intervention with ascorbic acid supplementation, smokers’ plasma α- and γ-tocopherol disappearance kinetics were restored to levels observed in nonsmokers. Collectively, these data indicate that cigarette smoking causes increased α- and γ-tocopherol oxidation, decreased α-tocopherol metabolism, and that smokers have higher vitamin E dietary requirements than nonsmokers which is highly related to plasma ascorbic acid.
5.1. VALUE OF LC/MS FOR CLINICAL TRIALS

The investigations presented in this dissertation have taken full advantage of the recent technological advances of LC/MS (liquid chromatography/mass spectrometry) in order to gain sensitivity as well as specificity of the analytes of interest. In recent years, LC/MS has emerged as a superior alternative to gas chromatography/mass spectrometry (GC/MS) for analytical and quantitative work at least as it relates to vitamin E. With specific regard to the analysis of vitamin E related compounds, LC/MS allows for quicker, more reliable and less costly sample preparation, since no sample derivitization is required (Lauridsen et al., 2001b), and enables investigators to more conveniently follow changes in plasma tocopherols using deuterium labeled tocopherols as was demonstrated in the two kinetics studies that are described in this dissertation. In addition, the analytical methods for vitamin E that have been developed for our LC/MS have a much higher throughput as demonstrated by total run times of ~6 min thus making the analysis of large scale clinical investigations timely and possible.
5.2. CIGARETTE SMOKING CAUSES $\gamma$-TOCOPHEROL NITRATION

The detection of NO$_2$-$\gamma$-tocopherol was a novel observation since no other investigations to date conducted in cigarette smokers have analyzed and quantitated this compound. Its detection at significantly higher concentrations among smokers compared to nonsmokers signifies that the reactive nitrogen species generated from the enhanced inflammatory response of smokers can directly react with $\gamma$-tocopherol. Given that $\gamma$-tocopherol is quite lipophilic and peroxynitrite often targets lipophilic biomolecules (Hensley et al., 2000), the routine measurement of NO$_2$-$\gamma$-tocopherol from biological fluids may enable its use as a biomarker for evaluating nitrosative stress and inflammation. Similarly, work has been conducted using nitro-tyrosine as a marker associated with increased odds of coronary artery disease (Shishehbor et al., 2003). However, literature suggests that the nitration of $\gamma$-tocopherol occurs ~15-times more readily than the nitration of tyrosine (Goss et al., 1999). Therefore, NO$_2$-$\gamma$-tocopherol may actually be a better biomarker for inflammation-related pathologies than nitro-tyrosine. Future research is clearly warranted in this area.
5.3. FASTER $\alpha$-TOCOPHEROL DEPLETION IN SMOKERS IS RELATED TO PLASMA ASCORBIC ACID

Following oral administration of deuterium labeled $\alpha$-tocopherols and subsequent LC/MS analysis of plasma samples, we found that cigarette smoking causes greater rates of plasma $\alpha$-tocopherol disappearance and shorter $\alpha$-tocopherol half-lives when compared to nonsmokers. Furthermore, since urinary $\alpha$-tocopherol metabolite ($\alpha$-CEHC) excretion at the end of the investigation was not increased among the smokers, but rather decreased, the greater $\alpha$-tocopherol losses were attributed to increased $\alpha$-tocopherol oxidation rather than increased metabolism. Moreover, during this investigation a correlation between $\alpha$-tocopherol disappearance rates and average plasma ascorbic acid was observed. However, this relationship was only observed among the smokers despite similar average plasma ascorbic acid concentrations between smokers and nonsmokers, a finding that suggests that both low ascorbic acid concentrations and oxidative stress are necessary to negatively influence $\alpha$-tocopherol depletion.

This investigation provided evidence that cigarette smoking can increase vitamin E utilization through oxidation pathways rather than metabolic pathways thus providing direct evidence that $\alpha$-tocopherol functions as an *in vivo* antioxidant. Previous trials that measured non-deuterated $\alpha$-tocopherol have only provided results that are ambiguous since some investigations report significantly lower plasma $\alpha$-tocopherol (Bolton-Smith et al., 1991; Mezzetti et al., 1995) whereas others have observed no differences (Dietrich et al., 2003; Lykkesfeldt et al., 2000) between
smokers and nonsmokers. Furthermore, previous kinetics studies (Munro et al., 1997; Traber et al., 2001) using deuterated tocopherols have been inconclusive as well. It was observed (Munro et al., 1997) that smokers had lower deuterium α-tocopherol plasma concentrations compared to nonsmokers at all time points that blood samples were collected, but limitations in the study design did not enable the investigators to discern if the differences in plasma concentrations were due to increased α-tocopherol utilization or less α-tocopherol absorption among the smokers. Furthermore, subsequent work by others (Traber, 1994) observed that smokers, on average, had faster plasma deuterium α-tocopherol disappearance compared to nonsmokers, but due to the small sample size of the investigation the differences were not statistically significant.
5.4. VITAMIN C INTERVENTION ATTENUATES VITAMIN E DEPLETION IN SMOKERS

Based on the observation that α-tocopherol disappearance rates and plasma ascorbic acid concentrations in smokers were correlated, we conducted a short-term, double-blind, placebo-controlled, cross-over investigation that provided supplemental ascorbic acid (2-weeks, twice daily with 500 mg) or placebo to determine if increasing plasma ascorbic acid concentrations could effectively attenuate deuterated tocopherol disappearance. In this investigation, it was expected that ascorbic acid supplementation would reduce the magnitude of oxidative stress as marked by plasma F2α-isoprostanes, but no reduction in lipid peroxidation was observed in either the smokers or nonsmokers. Although ascorbic acid supplementation doubled plasma ascorbic acid concentrations in both smokers and nonsmokers, plasma α-tocopherol and γ-tocopherol disappearance rates were reduced by 24% and 30%, respectively, only among the smokers. The change in the rates could be a result of increased metabolism or decreased oxidation. The decreased disappearance rates of deuterated tocopherols in this investigation were not attributed to increased metabolism as demonstrated by unchanged disappearance rates of d2-γ-CEHC and the lack of detection of d6-α-CEHC or any changes in unlabeled plasma tocopherols. Therefore, it was concluded that ascorbic acid supplementation increased plasma ascorbic acid sufficiently to enable the reduction of tocopheroxyl radicals generated from the reaction between tocopherols and the peroxyl radicals that are associated with elevated lipid peroxidation.
While it has been speculated for some time that vitamin E and C can interact and numerous reports have demonstrated their interaction \textit{in vitro}, this is the first report in humans that provides direct evidence for this interaction \textit{in vivo}. The results of this investigation relate well to observations noted in the Antioxidant Supplementation in Atherosclerosis Prevention (ASAP) study (Salonen et al., 2000). The ASAP study demonstrated that combination supplementation with vitamins E (91 mg) and C (250 mg) for 3-years had the greatest effect in significantly reducing the progression of common carotid atherosclerosis in men who smoke whereas individual nutrient supplementation provided no benefit in any of the study groups. For comparison, the results from only vitamin E intervention trials have been conflicting as noted by the lack cardiovascular benefit observed in the GISSI (Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto miocardico)-Prevenzione (1999) or Heart Outcomes Prevention Evaluation (HOPE) (Yusuf et al., 2000) trials whereas the CHAOS trial (Cambridge Heart Antioxidant Study) (Stephens et al., 1996) demonstrated that vitamin E supplementation reduced subsequent nonfatal myocardial infarctions by 75%. Therefore, from a public health standpoint, it appears that maximal cardiovascular benefits are attained by the synergistic effects of vitamins E and C ingested together rather than from vitamin E or vitamin C supplementation alone.
5.5. FUTURE RESEARCH

Based on the work presented in this dissertation there are several areas related to vitamin E utilization that require future work. First and foremost, better dietary requirements for vitamin E need to be elucidated. The current dietary vitamin E requirements are based on work conducted more than 40 years ago that evaluated the \textit{in vitro} assessment of peroxide-induced hemolysis of erythrocytes obtained from men restricted to a low vitamin E diet for several years. Once more accurate dietary vitamin E recommendations are determined for nonsmokers, dietary vitamin E requirements for smokers can be extrapolated. Secondly, additional investigations are necessary to further understand the value of CEHCs as an \textit{in vivo} marker for vitamin E status. Since excessive vitamin E is metabolized by a hepatic cytochrome P450 dependent pathway it would seem plausible that lower vitamin E status would be associated with lower plasma and urinary CEHC. Likewise, since little is known about the regulation of these tocopherol metabolic pathways, additional work is warranted in that area as well. Lastly, future investigations are needed to determine if higher plasma ascorbic acid concentrations can protect $\gamma$-tocopherol from nitration and if supplementation with $\gamma$-tocopherol in human models of inflammation offers any health benefit.
In conclusion, it should always be recommended to smokers to cease smoking rather than formulate dietary recommendations that will help them to cope with the magnitude of oxidative stress experienced. However, in the past decade, the prevalence of smoking in the United States has not subsided (Mokdad et al., 2004) despite increased public awareness of the health risks associated with smoking. Therefore, based on the investigations found in this dissertation, it is only prudent to suggest that smokers attain at least the dietary vitamin E required by nonsmokers (15 mg/d α-tocopherol). In addition, because vitamin E utilization is strongly related to plasma ascorbic acid concentration, smokers should also strive to consume the RDA for ascorbic acid (125 and 110 mg/d for men and women, respectively) since the data from our study (Chapters 3 and 4) directly support higher dietary ascorbic acid intakes for smokers in order to decrease vitamin E disappearance rates to those observed in nonsmokers.
Figure 5.1. Overall effects of cigarette smoking on vitamin E utilization
Cigarette smoke results in oxidative stress marked by increased lipid peroxidation and reactive nitrogen species. Tocopherols can react with peroxyl radicals to cease the lipid peroxidation chain reaction but get oxidized in the process. Ascorbic acid can directly interact with tocopheroxyl radicals and reduce them to tocopherols. Reactive nitrogen oxides generated from inflammatory process attributed to cigarette smoke can directly react with $\gamma$-tocopherol to form 5-nitro-$\gamma$-tocopherol. However, cigarette smoking does not increase tocopherol metabolism.

Abbreviations: R', carbon-centered radical; ROO', peroxyl radical; ROOH, lipid hydroperoxide; RH, polyunsaturated fatty acid; NO$_x$, reactive nitrogen oxides; NGT, 5-nitro-$\gamma$-tocopherol; T, tocopherols; T', tocopheroxyl radical; AA, reduced form ascorbic acid; AA', oxidized form of ascorbic acid.
LIST OF REFERENCES


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APPENDIX

CONSENT FORMS
CONSENT TO PARTICIPATE IN A RESEARCH STUDY

A. TITLE OF THE RESEARCH PROJECT

Oxidative Stress and Vitamin E Requirements – Trial 1

B. INVESTIGATORS

| Maret G. Traber, PhD – Principal Investigator | Richard S. Bruno, MS, RD
| Professor | Faculty Research Assistant
| Department of Nutrition and Food Management | Department of Nutrition and Food Management
| Linus Pauling Institute | Linus Pauling Institute
| 571 Weniger Hall | 561 Weniger Hall
| Oregon State University | Oregon State University
| Corvallis, OR 97331 | Corvallis, OR 97331
| Phone: 541-737-7977 | Phone: 541-737-9476
| Fax: 541-737-5077 | Fax: 541-737-5077
| Email: maret.traber@oregonstate.edu | Email: richard.bruno@oregonstate.edu
C. PURPOSE OF THE RESEARCH PROJECT:

You are being asked to participate in a research study. From this study, we hope to learn whether smoking cigarettes destroys the bodies’ supply of vitamin E. For this study, we need both smokers and non-smokers. If you do not smoke, we do not want you to smoke. If you smoke, or have smoked in the past, you will be asked to discontinue smoking for 1 hour prior blood drawing. You may be one of 24 subjects enrolled in this study.

D. PROCEDURES

1. PRE-STUDY SCREENING

You have been asked to come to this pre-study screening to learn about our study and to provide a fasting blood sample. If you have eaten anything besides water for the past 12 hours, you are not eligible to donate blood. After you have read this form and signed it, we will measure your blood pressure, your height and weight, and a blood sample will be drawn to measure your blood cell count and whether you have normal levels of blood cholesterol and enzymes. If you have normal blood chemistry and are healthy and fit our study criteria, we will call you within 1 week to invite you to participate in our vitamin E study. It may take up to 6 months before we assign you to a participation group.

2. WHAT PARTICIPANTS WILL DO DURING THE STUDY

- If you decide to volunteer, we will provide you a container to collect your urine for 24 hours before you come to the Center for your first meal. We will also provide a Styrofoam container filled with “blue ice” to keep your urine cold.

- We will invite you to the Nutrition Research Center to eat dinner daily for 1 week.

- Everyday for 1 week, we will provide you a capsule to eat at the end of your dinner. This capsule contains vitamin E that been specially tagged to make it heavier, so we can measure it. The vitamin E is NOT radioactive and is not harmful in anyway. Except for the label, it is identical to the vitamin E you purchase at the drug store.

- At the end of the first week, we will provide another container for you to collect your urine for 24 hours.

- Every morning for the first 2 weeks, we will ask you to return to the Center early in morning (about 7 AM) after fasting for 12 hours. We will then take a blood sample from a vein in your arm. A needle will be inserted in
the vein and 15 ml (1 tablespoon) of blood will be removed. We will then provide you a breakfast.

- Then, we will ask you to come to the center 3 times a week for the next 2 weeks (A total of 18 blood samples will be taken). On 5 days throughout the study that you provide a blood sample, we will remove an additional 15ml of blood. The total blood drawn will be less than a cup and a half (345 ml).

- At the end of the study, we will provide another container for you to collect your urine for 24 hours.

- A typical schedule for the study is shown on the following page:
<table>
<thead>
<tr>
<th>Week</th>
<th>Sunday</th>
<th>Monday</th>
<th>Tuesday</th>
<th>Wednesday</th>
<th>Thursday</th>
<th>Friday</th>
<th>Saturday</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6PM: start urine collection</td>
<td>6PM: end urine collection</td>
<td>8AM: fasting blood sample, breakfast</td>
<td>8AM: fasting blood sample, breakfast</td>
<td>8AM: fasting blood sample, breakfast</td>
<td>8AM: fasting blood sample, breakfast</td>
<td>8AM: fasting blood sample, breakfast</td>
</tr>
<tr>
<td></td>
<td>7PM: vitamin E pill with dinner</td>
<td></td>
<td>7PM: vitamin E pill with dinner</td>
<td>7PM: vitamin E pill with dinner</td>
<td>7PM: vitamin E pill with dinner</td>
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</tr>
<tr>
<td>2</td>
<td>8AM: fasting blood sample, breakfast</td>
<td>8AM: fasting blood sample, breakfast</td>
<td>8AM: fasting blood sample, breakfast</td>
<td>8AM: fasting blood sample, breakfast</td>
<td>8AM: fasting blood sample, breakfast</td>
<td>8AM: fasting blood sample, breakfast</td>
<td>8AM: fasting blood sample, breakfast</td>
</tr>
<tr>
<td></td>
<td>6PM: start urine collection</td>
<td>6PM: end urine collection</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
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</tr>
<tr>
<td>3</td>
<td>8AM: fasting blood sample, breakfast</td>
<td>8AM: fasting blood sample, breakfast</td>
<td>8AM: fasting blood sample, breakfast</td>
<td>8AM: fasting blood sample, breakfast</td>
<td>8AM: fasting blood sample, breakfast</td>
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<tr>
<td>4</td>
<td>8AM: fasting blood sample, breakfast</td>
<td>8AM: fasting blood sample, breakfast</td>
<td>8AM: fasting blood sample, breakfast</td>
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<td>8AM: fasting blood sample, breakfast</td>
<td>8AM: fasting blood sample, breakfast</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6PM: start urine collection</td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>6PM: end urine collection</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
3. **FORESEEABLE RISKS OR DISCOMFORTS**

Risks of blood drawing include some discomfort, bruising, and rarely infection. Only persons experienced in taking blood (licensed phlebotomists) will draw blood.

4. **BENEFITS TO BE EXPECTED FROM THE RESEARCH**

Results of this study will increase our knowledge about how cigarette smoking affects antioxidant nutrients. You will not be monetarily compensated for the initial screening blood draw, but you will learn of your blood testing results as part of our screening study. You will be paid for the samples of blood and urine we take during the study and you will be provided breakfast.

E. **CONFIDENTIALITY**

The results from this study will be published in the scientific literature. The identity of the subjects will be kept confidential; only a code will be published. Absolute confidentiality cannot be guaranteed, since research documents are not protected from subpoena.

F. **COMPENSATION**

After the initial screening, you will receive $10 per blood draw (18 draws, for $180). You will receive $15 for each of the 24 hour complete urine collections (3 urine collections, for $45). If you complete the study, providing all of the blood samples and the 3 urine samples you will receive a bonus of $50, for a total of $275.

G. **COSTS**

Study participants will not be assessed any charges for their participation in this study.

H. **VOLUNTARY PARTICIPATION STATEMENT**

You may change your mind about being in the study and withdraw after the study has started. Your participation is completely voluntary and you may either refuse to participate or withdraw from the study at any time without penalty or loss of benefits to which you are otherwise entitled. If you withdraw from the study before it is completed, the amount of money will be less than the full amount.
I. PRINCIPAL INVESTIGATOR’S DISCLOSURE OF PERSONAL OR FINANCIAL INTERESTS IN THE RESEARCH STUDY AND SPONSOR

Your investigators have NO financial interest in this research.

J. RESEARCH RELATED INJURY

In the event of research related injury, compensation and medical treatment is not provided by Oregon State University.

K. QUESTIONS

If you have any questions, please ask us now. If you have any additional questions later concerning the research study or specific procedures, contact Richard Bruno (541-737-9476 or richard.bruno@oregonstate.edu), who will answer them. If you have any questions about your rights as a research participant, please contact the OSU Institutional Review Board (IRB) Coordinator at (541) 737-3437 or by e-mail at IRB@oregonstate.edu.

L. CONSENT

YOUR SIGNATURE BELOW WILL INDICATE THAT YOU HAVE DECIDED TO VOLUNTEER AS A RESEARCH SUBJECT AND THAT YOU HAVE READ AND UNDERSTOOD THE INFORMATION PROVIDED ABOVE.

______________________________  __________________________
Signature of Participant or Legal Representative    Date

______________________________
Subject’s Printed Name

______________________________  __________________________
Signature of Investigator    Date

You will be given a signed and dated copy of this form to keep.
CONSENT TO PARTICIPATE IN A RESEARCH STUDY

A. TITLE OF THE RESEARCH PROJECT

Oxidative Stress and Vitamin E Requirements – Trial 3

B. INVESTIGATORS

<table>
<thead>
<tr>
<th>Maret G. Traber, PhD – Principal Investigator</th>
<th>Richard S Bruno, MS, RD Faculty Research Assistant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Department of Nutrition and Food Management</td>
<td>Department of Nutrition and Food Management</td>
</tr>
<tr>
<td>Linus Pauling Institute</td>
<td></td>
</tr>
<tr>
<td>561A Weniger Hall</td>
<td>Linus Pauling Institute</td>
</tr>
<tr>
<td>Oregon State University</td>
<td>561 Weniger Hall</td>
</tr>
<tr>
<td>Corvallis, OR 97331</td>
<td>Oregon State University</td>
</tr>
<tr>
<td>Phone: 541-737-7977</td>
<td>Phone: 541-737-9476</td>
</tr>
<tr>
<td>Fax: 541-737-5077</td>
<td>Fax: 541-737-5077</td>
</tr>
<tr>
<td>Email: <a href="mailto:maret.traber@oregonstate.edu">maret.traber@oregonstate.edu</a></td>
<td>Email: <a href="mailto:richard.bruno@oregonstate.edu">richard.bruno@oregonstate.edu</a></td>
</tr>
</tbody>
</table>
C. PURPOSE OF THE RESEARCH PROJECT:

You are being asked to participate in a research study. The purpose of this study is to evaluate the effects of vitamin C supplementation and cigarette smoking on vitamin E turnover kinetics in smokers and non-smokers. This study will assess if cigarette smoking increases vitamin E utilization and if vitamin C decreases vitamin E utilization. For this study, we need both smokers and non-smokers. If you do not smoke, we do not want you to smoke. If you smoke, you will be asked to discontinue smoking for 1 hour prior blood drawing. You may be one of 40 subjects enrolled in this study.

D. PROCEDURES

1. PRE-STUDY SCREENING

You have been asked to come to this pre-study screening to learn about our study and to provide a fasting blood sample. If you have eaten or consumed anything other than water for the past 12 hours, you are not eligible to give a screening blood sample at this time. After you have read this form, signed it, and if you are in a fasting condition we will measure your blood pressure, your height and weight, and a blood sample will be drawn to measure your blood cell count and whether you have normal levels of blood cholesterol and enzymes. If you have normal blood chemistry and are healthy and fit our study criteria, we will call you within 1 week to invite you to participate in our vitamin E study. It may take up to 6 months before we assign you to a participation group. However, based on similarly performed studies conducted in our laboratory, participation group assignment typically takes about 1-2 weeks following the pre-screening.

2. WHAT PARTICIPANTS WILL DO DURING THE STUDY

• If you choose to volunteer for this study, we will provide you with nutritional supplements (vitamin C or placebo). You will be asked to take these supplements each day throughout the study (2 pills per day of 500 mg; taken in the morning and evening with meals). This dose of vitamin C is what you might obtain from a typical over the counter supplement purchased at your local health food store. The dose is between the lower and upper recommendations of 75-2000 mg/day set forth by the United States Food and Nutrition Board.

• After 2-weeks, you will return to the Nutrition Research Center to eat breakfast on a single occasion. At that time, we will provide to you a capsule to eat at the end of your meal. This capsule contains vitamin E that has been specially tagged to make it heavier, so we can measure it. The vitamin E is NOT radioactive and is not harmful in any way. Except for the label it is identical to the vitamin E you purchase at the drug store.
• We will then collect blood at 0, 3, 6, 9, 12, 24, 36, 48 and 72 hours. For each blood sample, 22 ml (1.5 tablespoons) of blood will be collected for a total of 198 ml (just under 1-cup). In addition, urine will be collected from 24 to 48 h with the provided urine containers.

• After approximately 3 months, you will be invited back to the study center to complete the alternative treatment of the trial (vitamin C or placebo). The same procedures will take place at that time.

3. **FORESEEABLE RISKS OR DISCOMFORTS**

Risks of blood drawing include some discomfort, bruising, and rarely infection. Only persons experienced in taking blood (licensed phlebotomists) will draw blood. No adverse effects should be experienced as a result of vitamin C supplementation (500 mg, twice daily). Vitamin C is a non-toxic, water-soluble vitamin and excesses are readily excreted in urine. The recommended upper limit for vitamin C is 2000 mg/day and published reports suggest that adverse effects such as gastrointestinal disturbances may occur in certain individuals at doses between 3-10 grams of vitamin C daily.

4. **BENEFITS TO BE EXPECTED FROM THE RESEARCH**

There are no direct benefits to participants, other than learning about their initial blood testing results. You will not be monetarily compensated for the initial screening blood draw, but you will learn of your blood testing results as part of our pre-screening. You will be paid for the samples of blood and urine we take during the study and you will be provided breakfast. Results of this study will increase our knowledge about how cigarette smoking affects antioxidant nutrients.

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**E. CONFIDENTIALITY**

The results from this study will be published in the scientific literature. Your identity will be kept confidential to the extent permitted by law; only a code will be published. Absolute confidentiality cannot be guaranteed, since research documents are not protected from subpoena.
F. COMPENSATION

For each phase of the study (placebo and vitamin C supplementation), you will be compensated in the following manner:

- $10 per blood draw after vitamin E administration (0, 3, 6, 9, 12, 24, 36, 48 and 72 h); a total of $90 if all of the samples are obtained.
- $15 per complete 24 h urine collection (1 complete urine collection, for a total for $15)
- Therefore, if you complete both phases of the study and provide all the blood and urine samples, then you will be compensated a total of $275 as outlined below.
  - $10 * 18 blood draws = $180
  - $15 * 2 24h urine collections = $30
  - Study Bonus = $65
  - Total = $275

G. COSTS

There are no costs to you for your participation in this study.

H. VOLUNTARY PARTICIPATION STATEMENT

You may change your mind about being in the study and withdraw after the study has started. Your participation is completely voluntary and you may either refuse to participate or withdraw from the study at any time without penalty or loss of benefits to which you are otherwise entitled. If you withdraw from the study before it is completed, the amount of money will be less than the full amount.

I. PRINCIPAL INVESTIGATOR’S DISCLOSURE OF PERSONAL OR FINANCIAL INTERESTS IN THE RESEARCH STUDY AND SPONSOR

Your investigators have NO financial interest in this research.
J. RESEARCH RELATED INJURY

In the event of research related injury, compensation and medical treatment is not provided by Oregon State University.

K. QUESTIONS

If you have any questions, please ask us now. If you have any additional questions later concerning the research study or specific procedures, contact Maret Traber (541-737-7977 or maret.traber@oregonstate.edu) or Richard Bruno (541-737-9476 or richard.bruno@oregonstate.edu), who will answer them. If you have any questions about your rights as a research participant, please contact the OSU Institutional Review Board (IRB) Human Protections Administrator at (541) 737-3437 or by e-mail at IRB@oregonstate.edu.

L. CONSENT

YOUR SIGNATURE BELOW WILL INDICATE THAT YOU HAVE DECIDED TO VOLUNTEER AS A RESEARCH SUBJECT AND THAT YOU HAVE READ AND UNDERSTOOD THE INFORMATION PROVIDED ABOVE.

Signature of Participant or Legal Representative Date

Participant’s Printed Name

Signature of Investigator Date

You will be given a signed and dated copy of this form to keep.