AN ABSTRACT OF THE THESIS OF

Angela Mastaloudis for the degree of Master of Science in Nutrition and Food Management presented on August 23, 2000. Title: Assessment of Oxidative Stress in Athletes During Extreme Endurance Exercise Using Deuterium-labeled Vitamin E.

Abstract approved: _____ Maret G. Traber

To determine whether extreme endurance exercise induces lipid peroxidation, we studied 14 athletes (5 females: 9 males) during a 50 km ultramarathon (trial 1) and during a sedentary protocol (trial 2) one month later. At dinner the evening before the race (or sedentary trial), subjects consumed vitamin E labeled with stable isotopes (75 mg each d_3-*RRR* and d_6-*all rac*-α–tocopheryl acetates). Blood samples were taken at baseline, 30 minutes pre-race, mid-race, post-race, 1 h post-race, 24 h post-race, and at corresponding times for each individual during trial 2. During the sedentary day of trial 2, subjects consumed the same amounts of race day foods, including ergogenic aids, that they had consumed in trial 1; vitamin E intakes were 77 ± 40 mg, and vitamin C 406 ± 169 mg. All 14 subjects completed the race; average time to completion was 390 ± 67 minutes. Plasma F_2-isoprostanes (F_2-I), labeled and unlabeled α-tocopherol, and ascorbic acid (AA) were measured. F_2-I increased from 76 ± 24 pg/ml pg/ml at pre-race to 117.4 ± 38.0 pg/ml ($p<0.0008$) at mid-race to 130 ± 54 pg/ml ($p<0.0001$) at post-race, then returned to baseline at 24 hours post-race; F_2-I were unchanged during trial 2. Deuterated d_3 α-tocopherol (d_3 α-Toc) disappearance rates were faster ($2.8x10^{-4} \pm 0.5x10^{-4}$) during the race compared to the sedentary trial ($2.3x10^{-4} \pm 0.6x10^{-4}$; $p < 0.03$). Plasma AA *increased* from 75.2 ± 11.2 μM at pre-race to 157.7 ± 36.2 μM at race end ($p<0.0001$) and decreased to below baseline concentrations at 24 h post-race (40.4 ± 5.2 μM $p<0.0001$).

AA levels also increased during trial 2. Despite increased plasma AA, F_2-I increased during exercise, but not during the sedentary period. Additionally, vitamin E disappeared faster during the run compared to the sedentary protocol. Thus, extreme endurance exercise results in the generation of lipid peroxidation and increased vitamin E utilization.

Assessment of Oxidative Stress in Athletes During Extreme Endurance Exercise Using Deuterium-labeled Vitamin E

by

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A THESIS

Submitted to

Oregon State University

In partial fulfillment of
the requirements for the degree of

Master of Science

Presented August 23, 2000
Commencement June 2001

Master of Science thesis of Angela Mastaloudis presented on August 23, 2000

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

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Angela Mastaloudis, Author

CONTRIBUTION OF AUTHORS

Special thanks to Dr. James E Leklem, with whom this was a collaborative project. His lab was responsible for the design and implementation of the controlled diets as well as the logistics for the ultramarathon race. Scott Leonard was involved in the sample collection and data analysis for this study. Dr. Maret G. Traber supervised the design analysis, interpretation and writing of the study.

Dr. Charlotte Lauridsen and Dr. John Lodge helped with sample collection. Deborah Hobbs trained me for the F_2-isoprostanes assay. Thanks to Dr. Balz Frei for sharing his expertise on Vitamin C and to Dr. Jeff Widrick and Dr. Dan Williams for their expert advice concerning the exercise components of the study.

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1. INTRODUCTION

The *hypothesis* tested was that exercise increases oxidative stress. This increased oxidative stress should decrease circulating antioxidant concentrations, increase vitamin E turnover, and increase lipid peroxidation. Extreme exercise was chosen as a model to test whether physical activity causes in vivo oxidative stress. The effects of extreme exercise in trained individuals during an ultramarathon and then in the same individuals during a sedentary trial were compared.

Specific Aim: to test in athletes performing an ultramarathon compared to a sedentary period, if:

- vitamin E utilization is increased
- plasma markers of lipid peroxidation are increased
- circulating antioxidants are decreased

By taking advantage of the most recently developed techniques for assessing lipid peroxidation, i.e. vitamin E biokinetics, F_2-Isoprostanes, and tracking circulating antioxidants, the proposed study has helped us answer the question of whether extreme endurance exercise results in oxidative stress with much greater certainty than previous studies.

2. BACKGROUND AND SIGNIFICANCE

During the resting state, the human body produces reactive oxygen species (ROS) including superoxide, hydrogen peroxide and hydroxyl radicals, but at levels well within the capacity of the body's antioxidant defense system. The most important source of superoxide anions in humans may be the electron transport chain of the mitochondria (1). Under normal conditions, approximately 1-3% of electrons leak from the mitochondria (1), resulting in formation of superoxide. The rate of leakage is thought to increase with increases in oxygen concentration (1). Considering that during endurance exercise there is a 10-20 fold increase in whole body oxygen consumption (2), and oxygen uptake in the active skeletal muscle increases 100-200 fold (1, 3), it is likely that increased oxygen utilization during exercise results in the production of reactive oxygen species at rates that exceed the body's capacity to detoxify them (4). This may lead to the accumulation of excess amounts of reactive oxygen species, which can in turn cause lipid peroxidation, as well as nucleic acid and protein oxidation (5). Since vitamin E is the antioxidant primarily responsible for scavenging peroxyl radicals (6), this increase in lipid peroxidation may also deplete vitamin E. In the 1980's, Davies et al (7) conducted one of the earliest studies addressing the issue of oxidative stress associated with exercise. They reported in male Long Evans rats that exercise greatly increased lipid peroxidation, as measured using the TBARS assay (a measure of malondialdehyde). Since that time, studies have been carried out in humans to evaluate whether oxidative stress occurs as a result of endurance exercise. Some studies in humans have supported Davies' findings (8-13), while others have refuted them (14-17).

2.1. SOURCES OF REACTIVE OXYGEN SPECIES

2.1.1. Mitochondrial Electron Transport Chain

The electron transport chain of the mitochondria is not entirely efficient as there is an approximate 1-3% leakage of electrons from the electron transport chain (1). What causes the electron leakage, and what are the implications? The mitochondrion is unique in two ways: it has two cell membranes rather than one and it has its own DNA and some of its own proteins (18). The electron transport chain (ETC) is embedded within the inner membrane of the mitochondria; it is the primary site in the cell for oxidative phosphorylation. Oxidative phosphorylation produces ATP, the primary energy source for cells.

The electron transport chain is composed of four protein complexes; complexes I, III and IV are the locations of the proton pumps. In complex I, NADH is oxidized to NAD^+, coenzyme Q (CoQ), also known as ubiquinone, is the electron (e^-) acceptor. In the process, coenzyme Q is reduced to the semiquinone radical, which can in turn accept a second electron to form ubiquinol ($CoQH_2$), a completely reduced molecule (1). Complex II involves the oxidation of $FADH_2$ to FAD and also uses coenzyme Q as its e^- acceptor. Ubiquinol delivers electrons to complex III and finally, electrons are transferred from there to complex IV by cytochrome c. Complex IV, also known as cytochrome oxidase, is the intended site for the transfer of two electrons to molecular oxygen to form H_2O. It has been established, though, that in the process of any of these transfers, electrons may inadvertently be transferred to molecular oxygen in the intermembrane space, rather than to their intended receptor molecule (19). Semiquinone has been the most implicated in the donation of an electron to molecular oxygen (19). Regardless of the mechanism of transfer, when a single e^- is transferred to

molecular oxygen, the superoxide anion ($O_2^{\bullet -}$), a reactive oxygen species (ROS), is formed in a reduction reaction. If superoxide does not react with other molecules present, it rapidly dismutates to hydrogen peroxide (H_2O_2), a reaction that may occur non-enzymatically or may be catalyzed by the enzyme superoxide dismutase (20). Although hydrogen peroxide is relatively non-reactive, it can be cytotoxic at high concentrations e.g. 10-100 μM range (1), as it can oxidize specific keto-acids such as pyruvate and can inactivate certain enzymes such as glyceraldehyde-3-phosphate dehydrogenase. More importantly, hydrogen peroxide is non-polar and thus capable of crossing plasma membranes and traveling to other parts of the cell, increasing the likelihood that it will encounter free metals such as iron. In the presence of free metals, hydrogen peroxide can react to form the highly reactive hydoxyl radical ($^{\bullet}OH$) (see below). Thus, hydrogen peroxide can lead to cell damage at a distance relatively far from the original source of reactive oxygen species.

2.1.2. Metal-catalyzed Reactive Oxygen Species Chemistry

Under normal physiological conditions iron is tightly regulated and most, if not all iron, is bound to proteins such as transferrin or ferritin (21). Bound iron is non-reactive, but there also exists a small pool of low molecular mass iron that is thought to participate in redox cycling. Unbound iron can react with hydrogen peroxide in the Fenton Reaction (1) to form the very reactive hydoxyl radical ($^{\bullet}OH$).

$$H_2O_2 + Fe^{2+} \rightarrow Fe\ (III) + OH^- + {}^{\bullet}OH \quad k_2 = 76\ M^{-1}\ s^{-1}$$

$^{\bullet}OH$ is the most reactive of the reactive oxygen species and has the potential to damage DNA, lipid membranes and proteins (1).

Another reaction by which iron and hydrogen peroxide can form hydroxyl radicals is the iron-catalyzed Haber-Weiss (22) reaction:

$$\text{Fe (III)} + O_2^{\bullet -} \rightarrow Fe^{2+} + O_2$$

$$Fe^{2+} + H_2O_2 \rightarrow OH^- + {}^{\bullet}OH + \text{Fe (III)} \quad (1).$$

$$\text{Net: } O_2^{\bullet -} + H_2O_2 \rightarrow O_2 + {}^{\bullet}OH + OH^- \qquad \text{(Haber-Weiss reaction)}$$

The amount of H_2O_2 accumulating in vivo is minimized by the enzyme catalase.

$$\text{catalase-Fe(III)} + H_2O_2 \rightarrow \text{compound I} + H_2O$$

$$\text{compound I} + H_2O_2 \rightarrow \text{catalase-Fe (III)} + H_2O + O_2 \quad (1)$$

2.1.3. Sources of Transition Metal Catalysts in Exercise

A number of potential causes of free metal accumulation exist during exercise; low molecular mass iron (redox active) has been the most studied. According to Jenkins et al (21) exercise impacts tissue chemistry in a manner that increases the availability of low molecular mass iron. Furthermore, blood pH drops in response to exercise due to the accumulation of lactate and H^+ (23), and this low pH helps to maintain iron ions in solution (21) making them more readily available to react with free radicals. During distance running, intravascular hemolysis (mechanical trauma to red blood cells) occurs at foot strike as evidenced by decreased haptaglobin levels and increased plasma free hemoglobin (24). The extent of hemolysis increases with increased race distance (25). Damaged erythrocytes can provide a potential source of low molecular mass iron in the plasma (21).

Exercise induced muscle damage results in the release of relatively large amounts of myoglobin, a heme containing protein and a potential source of low molecular mass iron, into the plasma (26, 27). Exercise induced muscle damage may also cause alterations in the structure and function of the sarcoplasmic reticulum leading to compromises in calcium (Ca^{2+}) regulation (28, 29). The resulting calcium overload may induce calcium-activated proteases and phospholipases, which can break down membranes and proteins (28), potentially mobilizing low molecular mass iron in the process. Further evidence supporting the theory that exercise increases the prevalence of low molecular mass iron is the appearance of transition metals in sweat (21) and the occurrence of gastrointestinal blood loss associated with endurance exercise (30).

In healthy people, there are a number of defense mechanisms in place to mitigate oxidative damage, including superoxide dismutase, glutathione peroxidase, and catalase, but they are not 100% efficient. It may be that this inefficiency leads to minute amounts of free radical catalyzed damage in the cell, which over time causes impairment of cell function. The consequence of that damage may be that more free iron is allowed to move freely into the cytosol, amplifying oxidative damage in the membrane and beyond.

2.1.4. Potential Role of Xanthine Oxidase

The 10 to 20-fold increase in whole body oxygen consumption (2) and 100 fold (1) increase in oxygen uptake in the active skeletal muscle during endurance exercise reflects an increased energy requirement. This exercise associated increase in energy demand may exceed the capacity of the mitochondria to produce ATP via the electron transport chain rapidly enough, resulting in an accumulation of ADP. One alternate route of ATP production is the adenylate kinase system, which

operates within the cytosol. This mechanism converts 2 ADP molecules to 1 AMP + 1 ATP and is catalyzed by the enzyme adenylate kinase (4). While the resulting ATP is utilized for energy, it is the fate of AMP that is of special interest considering its potential contribution to oxidative stress during exercise. In the cytosol, AMP is rapidly converted to hypoxanthine, an uncharged molecule that can freely diffuse out of the muscle cells into the surrounding capillary epithelial cells (4). From that point hypoxanthine has two possible fates, both of which lead to the formation of uric acid.

In the resting state, hypoxanthine is converted to xanthine and subsequently to uric acid in a series of two reactions. Both reactions are catalyzed by xanthine dehydrogenase (XDH), an enzyme that utilizes NAD+ as its electron acceptor.

It has been hypothesized that during exercise, there may be insufficient ATP in the skeletal muscle cell to sustain the ATP-dependent calcium pumps, resulting in a build-up of calcium in the cytosol (4). Increased calcium may lead to increased activation of calcium activated proteases such as calmodulin and calpain (1, 4). Both of these proteases are known to catalyze the cleavage of a peptide from XDH (1) resulting in a conformational change in the enzyme; the resulting product is xanthine oxidase (XO). XO has the same function as XDH, the conversion of hypoxanthine to uric acid with xanthine as an intermediate product. The critical difference is that unlike XDH, XO utilizes molecular oxygen as its electron acceptor, forming the superoxide anion.

There may be times during endurance exercise that the cells' increased oxygen demand can not be met completely, and the system is unable to deliver oxygen to the skeletal muscles rapidly enough to meet the needs of the cell. In such cases localized hypoxia occurs (20). If XO is the primary form of that enzyme

in the cell, there will be a buildup of hypoxanthine, as there is not enough molecular oxygen available to act as an electron acceptor for this reaction to occur. Once exercise is ceased, and oxygen delivery resumes, hypoxanthine is rapidly converted to xanthine and subsequently uric acid. In this reaction, with XO as the catalyst, there is a rapid accumulation of the superoxide radical within the cell along with an increase in plasma levels of uric acid, a radical scavenger (31).

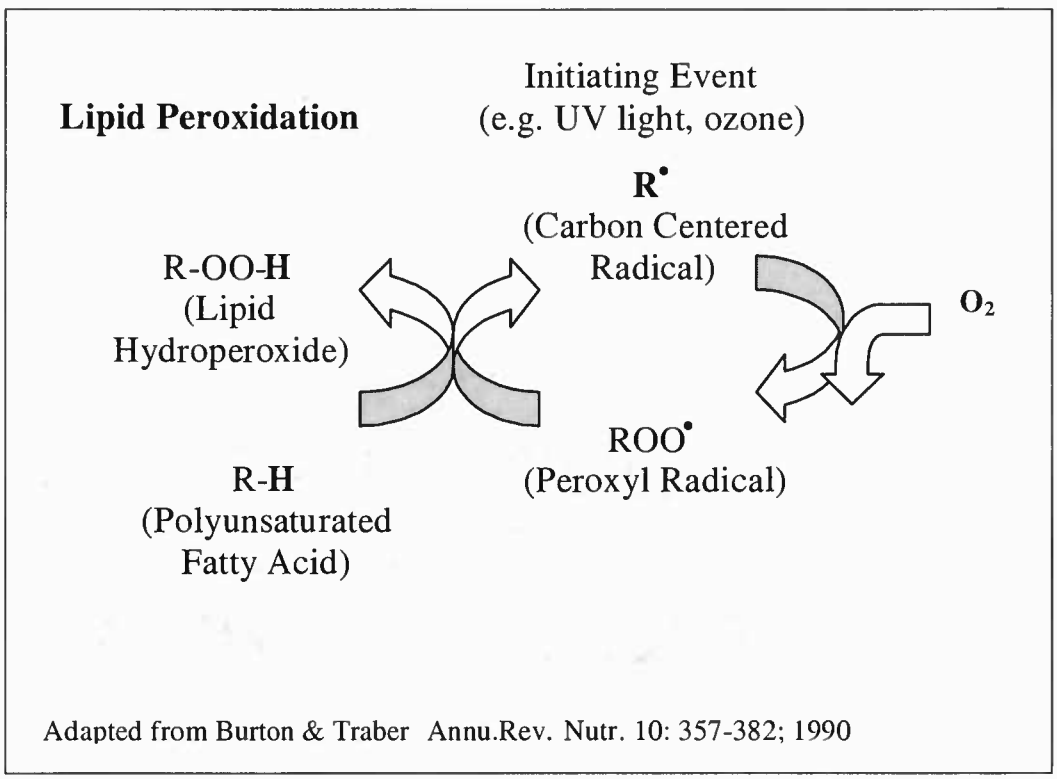
By incorporating a cool down period, an individual may be able to reduce the rate of reoxygenation of the skeletal muscle and consequently reduce the rate of conversion of hypoxanthine to xanthine and finally to uric acid by XO, thus producing superoxide at a slower rate. At a reduced rate of production, the accumulation of radicals may remain within the capacity of the body's antioxidant defense system, minimizing oxidative damage in the cell.

2.2. LIPID PEROXIDATION

Oxidative stress has been defined as a significant imbalance between reactive oxygen/reactive nitrogen species production and antioxidant defenses (1). This imbalance can be caused by an increased rate of reactive species production and/or decreased antioxidant production (32). Damage associated with oxidative stress (e.g. lipid peroxidation, DNA and, protein damage) is generally referred to as oxidative damage. Phospholipids are integral components of cell membranes and are essential for membrane fluidity and transport. Of the lipids commonly present in phospholipid membranes, polyunsaturated fatty acids (PUFA's) (fatty acids containing two or more double bonds) are most susceptible to lipid peroxidation. This is because of the presence of bis-allylic hydrogens; hydrogens located on a carbon that has double bonds on either side of it, –CH=CH–C**H**$_2$–CH=CH–, making the bonds weaker and thus more susceptible to abstraction. Oxidizability of

lipids increases proportionally with increases in the number of double bonds (33). A brief outline of lipid peroxidation is shown in Figure 1.

Figure 1. Lipid Peroxidation



Lipid peroxidation is a chain reaction initiated with abstraction of a hydrogen from a carbon by an initiating event such as UV light, ozone, radiation, pollution, etc., forming a carbon-centered radical ($R^{\bullet}$). The carbon-centered radical then undergoes a conformational change to form the more stable conjugated diene structure (1). In an aerobic environment, the most likely fate of the carbon-centered radical is reaction with molecular oxygen to form a peroxyl radical ($ROO^{\bullet}$) (1), a very reactive species. $ROO^{\bullet}$s can readily abstract a hydrogen from a bis-allylic hydrogen of a polyunsaturated fatty acid, creating both the lower energy lipid hydroperoxide (R-OO-H) and regenerating the carbon centered radical ($R^{\bullet}$), thus

perpetuating the chain of events. Termination may occur when two radicals react with one another (34), forming an inactive dimer: $R^{\bullet} + R^{\bullet} = R - R$. Alternatively, non-radical antioxidants may play a role in slowing the chain of events (see section on vitamin E below).

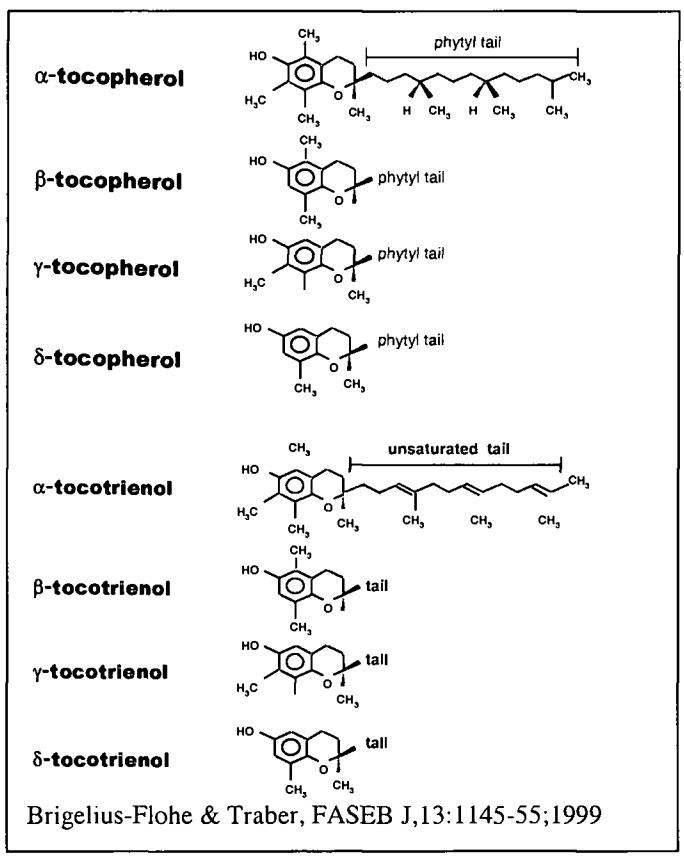
Lipid peroxidation can impair membrane fluidity and active transport of molecules and ions across the cell membrane (35). Additionally, lipid peroxidation products may initiate gene transcription or apoptosis, stimulate the immune response, cause inflammation, initiate fibrosis, or inactivate enzymes (36).

2.3. ANTIOXIDANTS

2.3.1. Vitamin E

Vitamin E is a fat-soluble micronutrient and as such, it is absorbed with fat via micelles at the intestine and transported in the plasma incorporated into chylomicrons and subsequently lipoproteins (6). Referring to Figure 2, the four tocopherols share a common saturated phytyl tail, but differ in the number of methyl groups on the chromanol ring. The term

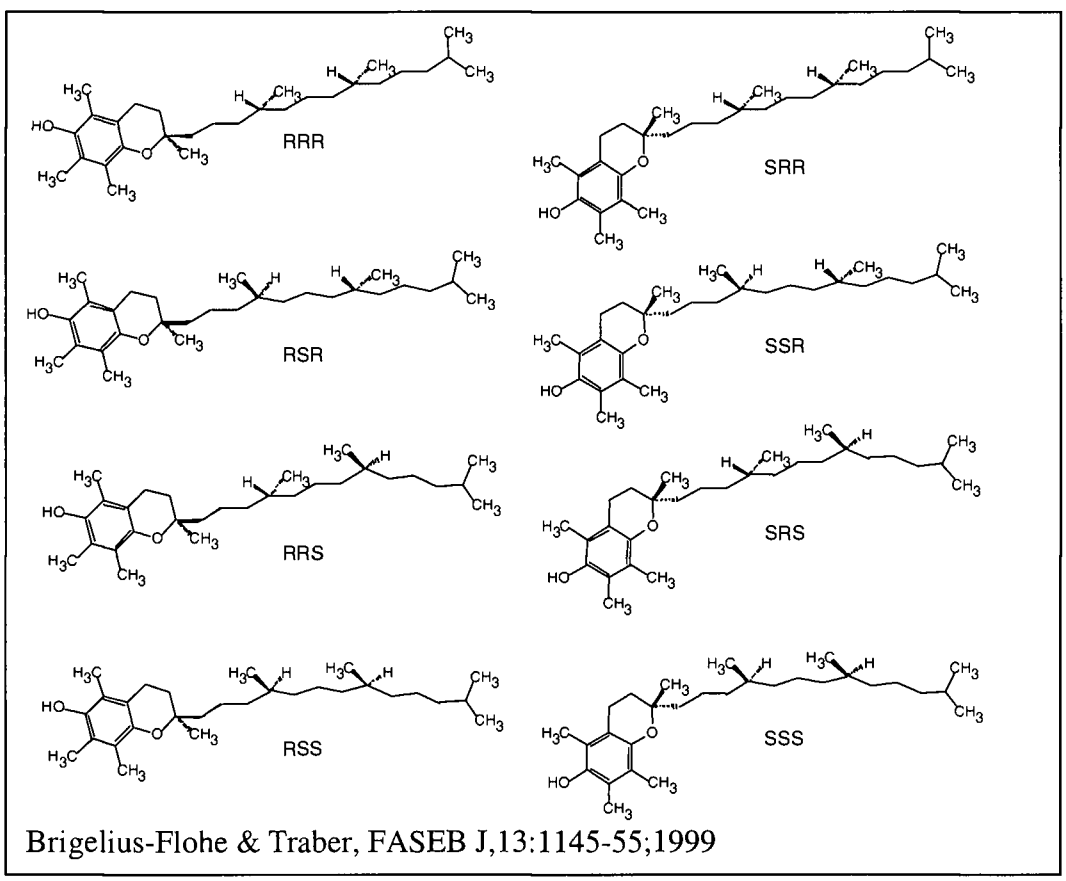
Figure 2. Vitamin E Structures



Brigelius-Flohe & Traber, FASEB J,13:1145-55;1999

vitamin E refers to the group of eight molecules including four tocopherols, α, β, γ, δ and four tocotrienols, α, β, γ, δ (37).

Figure 3. Synthetic Isomers of Vitamin E



Brigelius-Flohe & Traber, FASEB J,13:1145-55;1999

The tocotrienols differ from the tocopherols in that they have an unsaturated tail. Of these eight naturally occurring forms, α-tocopherol has the most antioxidant activity and is the most prevalent type found in nature (37). Supplements can contain the naturally occurring single stereoisomeric form, *RRR*-α-tocopherol, or synthetic *all-rac*-α-tocopherol. Synthetic vitamin E consists of eight stereoisomers of α-tocopherol that are distinct from one another due to differences at the three chiral centers of the phytyl tail (40), Figure 3.

Sources of vitamin E in the diet include: vegetable oils, especially wheat germ, safflower, sunflower, soybean olive and corn oils as well as nuts, sunflower seeds and whole grains (6, 38, 39). Although the Recommended Dietary Allowance for vitamin E is only 15 mg/day (38), larger doses of vitamin E are popular in the United States in the form of dietary supplements (6).

Supplements are typically sold as either acetate or succinate esters (40), since esterification prevents oxidation thus extending shelf life (38). These α-tocopheryl esters are hydrolyzed and absorbed with similar efficiencies (6). Importantly, *RRR*-α-tocopherol and *all-rac*-α-tocopherol are absorbed at the intestine and carried to the liver via chylomicrons without discrimination; nonetheless, *RRR*-α-tocopherol has twice the biological activity of the synthetic (38). This discrepancy is due to the preferential incorporation of *RRR*-α-tocopherol into very low-density lipoproteins (VLDL) by the α-tocopherol transport protein (α-TTP) in the liver (40, 41). Consequently, more *RRR*-α-tocopherol is delivered to the tissues by lipoproteins (40).

2.3.2. Vitamin C

Vitamin C, or ascorbic acid, is a water-soluble vitamin that has numerous functions in the body; it is a cofactor in the synthesis of collagen, carnitene and neurotransmitters among many others (38). Vitamin C is an essential vitamin because humans lack the functional enzyme L-gulonolactone oxidase required to synthesize ascorbate from glucose (42). Vitamin C is abundant in fruits and vegetables; some of the best sources include asparagus, papaya, oranges, and strawberries (43).

The term vitamin C refers to ascorbic acid, the reduced form and dehydroascorbic acid (DHA), the oxidized form of the vitamin (38). The biological

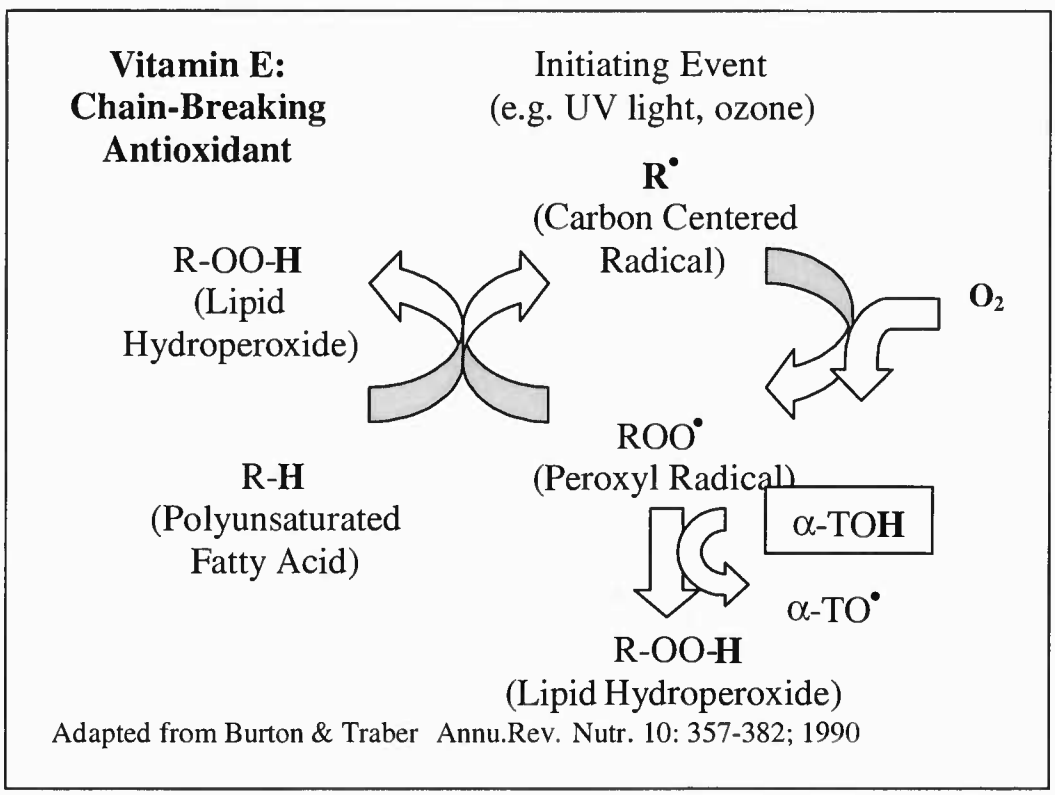
functions of ascorbic acid have been defined by its ability to provide reducing equivalents for critical reactions (38). One of the many roles of ascorbic acid is as an antioxidant, in fact, it is considered to be the most effective antioxidant in human plasma (44). Whereas other antioxidants such as vitamin E can greatly inhibit the rate of lipid peroxidation, ascorbate is the only antioxidant that can prevent initiation of lipid peroxidation induced by aqueous peroxyl radicals (45). Ascorbate belongs to the first line of antioxidant defenses. Not only does it prevent lipid peroxidation, it also spares other antioxidants such as α-tocopherol and urate (44). Although there is good evidence that vitamin C acts as a pro-oxidant in the presence of transition metals *in vitro*, there is not convincing evidence that vitamin C has pro-oxidant activity *in vivo* (42).

2.4. PLASMA ANTIOXIDANTS IN EXERCISE

Vitamin E (α–tocopherol) is a potent peroxyl scavenger and acts to protect polyunsaturated fatty acids (PUFA) within phospholipids of biological membranes against lipid peroxidation (6). α-Tocopherol can quench peroxyl radicals by donating a hydrogen, reducing the peroxyl radical to a lipid hydroperoxide, a lower energy molecule (Figure 4), but this does not terminate the chain reaction, as the α-tocopheroxyl radical is formed in the process. However, the α-tocopheroxyl radical is less reactive than the peroxyl radical and thus the chain of events is slowed (34). Notably, the reaction of a peroxyl radical with an α-tocopherol molecule occurs 1000 times faster than the reaction of the peroxyl radical with a polyunsaturated fatty acid, (38, 46).

Possible fates of α-tocopheroxyl radicals include: the radical can be further oxidized to the quinone (two electron oxidation), the radical can react with PUFA to form a peroxyl radical (pro-oxidant activity), the α-tocopheroxyl radical can react with another radical to form an adduct, two α-tocopheroxyl radicals can react with each other to form an inactive dimer, or the α-tocopheroxyl radical can be reduced by another antioxidant, such as ascorbate. Thus, vitamin C levels may change in response to direct or indirect oxidation. The response of plasma vitamin C to exercise has been increased levels (16, 17, 47), no change (48), or a reduction (49) in plasma ascorbic acid levels post-exercise.

Figure 4. Antioxidant Function of Vitamin E



Adapted from Burton & Traber Annu.Rev. Nutr. 10: 357-382; 1990

The effects of exercise on plasma levels of vitamins E and C has been examined by Viguie et al (16), who studied subjects exercising 90 minutes/day on a cycle ergometer for three consecutive days. They found no changes in plasma lipid hydroperoxides, urinary 8-hydroxyguanosine (a measure of oxidative RNA damage) or vitamin E plasma levels over the three day trial. Interestingly, they reported an increase in plasma ascorbic acid levels. Their study was of a shorter duration than the present study and involved concentric rather than eccentric exercise.

Although some researchers have looked at the effects of vitamin E supplementation on athletic performance/endurance (50, 51) and lipid peroxidation (52-54), none have used vitamin E biokinetics to evaluate oxidative stress. Previously, studies using deuterated α–tocopherols demonstrated that the various forms of vitamin E are absorbed and secreted similarly in chylomicrons, but that *RRR*- α–tocopherol is preferentially secreted in the liver as a result of the activity of the α–tocopherol transfer protein (α–TTP). Based on this mechanism, a mathematical model has been designed to determine the fractional disappearance rates of deuterated α–tocopherols (41). Our research has enabled us to compare the fractional rate of deuterated vitamin E disappearance during exercise and during a sedentary period in the same individuals, allowing us to evaluate whether vitamin E utilization increases during extreme exercise.

2.5. MARKERS OF LIPID PEROXIDATION

2.5.1. Malondialdehyde

The rate of free radical reactions is extremely rapid making it impractical to measure reactive oxygen species directly (55), therefore a number of indirect

markers have been used to assess lipid peroxidation. Previous methods used to assess lipid peroxidation such as the thiobarbituric acid reactive substances (TBARS) assay, have lacked sensitivity/specificity and are unreliable (56). Differences in the assays used to assess oxidative stress may account for much of the variability in previous studies. Malondialdehyde has been the most commonly used marker for lipid peroxidation in exercise studies and is often measured indirectly by TBARS. Malondialdehyde reacts with thiobarbituric acid to generate a colored product that is measured with a spectrophotometer. The main problems with this assay are that most TBARS detected are generated during sample preparation and the assay is non-specific in that thiobarbituric acid reacts with compounds other than malondialdehyde (1). This helps to explain why several similar studies involving trained male athletes running 21 km races, yielded opposite post-race results: an elevation (10, 13) or no change (11, 17) in TBARS.

2.5.2. Conjugated Dienes

Polyunsaturated fatty acid oxidation is accompanied by the formation of conjugated dienes, compounds that absorb UV light at 230-235 nm. Conjugated dienes measured spectrophotometrically have been used to assess lipid peroxidation. However, direct plasma conjugated diene measurements are not very reliable because other substances present, such as heme proteins, absorb strongly in the same UV range (1). A more useful conjugated dienes assay is to assess the susceptibility of low-density lipoproteins (LDL) to oxidation in vitro (1). However, LDL are susceptible to oxidation during the long centrifugation time required for isolation. Using LDL obtained from subjects who completed a half-marathon, Case et al (15) reported an *increase* in lag time. Conversely, using LDL from subjects after a full marathon Liu et al (12) reported a *decrease* in LDL lag time. More

puzzling, Marzatico et al (13) noted an increase in MDA levels after a half marathon, but no change in plasma conjugated dienes. These differences may be attributed to the duration of exercise, differences in antioxidant intakes or very likely, to technical difficulties.

2.5.3. F_2-Isoprostanes

Recently, a substantial body of evidence has accumulated indicating that the measurement of F_2-Isoprostanes is a sensitive, measure of in vivo lipid peroxidation (57) with a sensitive lower limit of detection in the low picogram range (58). F_2-Isoprostanes are unique, prostaglandin-like compounds produced by the non-enzymatic free-radical catalyzed oxidation of arachidonic acid (59). Arachidonic acid (20:4 n-6) is a long chain, polyunsaturated fatty acid and is a precursor for prostaglandins synthesis (18). F_2-Isoprostanes are structurally distinct from the prostaglandins (57) and unlike the prostaglandins, F_2-Isoprostanes are formed while arachidonic acid is still esterified to the phospholipid (60). Therefore, F_2-Isoprostanes can be quantified in vivo in both the esterified and free (non-esterified) forms. The mechanism of formation of F_2-Isoprostanes follows that of lipid peroxidation described previously. Initiation occurs with free radical initiated abstraction of a hydrogen atom from arachidonic acid forming an arachidonyl carbon-centered radical (60). Upon attack of the radical with molecular oxygen, a peroxyl radical is formed, followed by endocyclization and addition of a second oxygen molecule to form one of four bicycloendoperoxide regioisomers. These intermediates are then reduced to one of four possible regioisomers of F_2-Isoprostanes (60). Of these isomers, 8-epi-$PGF_{2\alpha}$ formation is favored (58, 59); it is also the compound most commonly measured in humans and it has been chemically synthesized (59). Importantly, 8-epi-$PGF_{2\alpha}$ has biological activity of it

own; it may act as potent vasoconstrictor and/or cause platelet aggregation (58). Detectable levels of F_2-isoprostanes have been found in all normal animal and human biological fluids and tissues tested thus far (60) and normal plasma levels for humans have been defined (57). This has allowed for the comparison of normal control subjects to test subjects and as such, F_2-isoprostanes have been shown to be elevated in the plasma of subjects with known oxidative stress, such as smokers and patients with cystic fibrosis and Alzheimer's disease (61-63).

2.6. ASSESSMENT OF OXIDATIVE STRESS IN ENDURANCE EXERCISE

There have been several major studies investigating the effects of distance running on oxidative stress in humans. The results have been inconsistent probably for the same reasons mentioned previously, differences in the exercise intensity, the training status of subjects, and the exercise environment. Variation in the assays used to assess oxidative damage was also likely a factor.

Maughan et al (8) studied normally physically active subjects during a 45 minute downhill treadmill run. Downhill running, an eccentric motion, is known to cause more muscle damage than running on a level surface or running uphill (27). They reported no increase in TBARS immediately post race, but by 6 hours post-race, levels had risen significantly. The rise in TBARS at 6 hours corresponded with a significant increase in plasma creatine kinase, a reliable marker of muscle damage (64), implying that in this case it was muscle damage inducing oxidative stress rather than a systemic response. These researchers concluded that there might be an association between the initiation of free radical reactions and the loss of membrane integrity responsible for the release of creatine kinase and other muscle-derived enzymes (8).

In contrast to the shorter term exercise bout used as a model by Maughan and colleagues, Inayama et al (14) reported no change in plasma TBARS immediately, 24 and 48 hours following a full marathon. However, the investigators did report a decline in plasma protein-bound sulfhydryl group values at race end indicating that the prolonged exercise caused oxidation of plasma proteins but not lipids (14). These are surprising results considering that the subjects were only moderately trained, inexperienced distance runners. The main difference may be that Maughan's study involved downhill running, which is known to cause extensive disruption of muscle fibers (27, 64). This supports the argument set forth previously that it is muscle damage that is inducing oxidative stress rather than a systemic response.

At least seven studies have assessed the amount of lipid peroxidation in response to a half-marathon run (22.1-km); despite using similar exercise protocols, these studies have yielded vastly conflicting results. Some investigators have reported increases in malondialdehyde following a half-marathon (10, 13) indicating that the exercise bout did result in lipid peroxidation. Other studies have been less conclusive. For example, Goodman et al (11) reported no change in malondialdehyde levels after the data had been corrected for plasma volume changes. However, they did find a significant increase in MDA in the uncorrected data. This group also took muscle biopsies; finding no evidence of ultrastructural damage associated with the elevated levels of MDA. These findings would argue that the oxidative stress was a systemic response, rather than a muscle damage response. Another possibility for the borderline increase in MDA is that there was increased oxidative stress, but not enough to cause damage. Finally, muscle biopsies are a very small sample from a large muscle mass and it is possible that

there was damage, but that the portion of the muscle taken in the biopsy did not include the damaged portion (65).

Another measure of lipid peroxidation, conjugated dienes, discussed previously, has been utilized in several half-marathon runs. Case et al (15) conducted one of the only studies involving women, and while two studies in male distance runners yielded no change in conjugated dienes (13, 17), her group reported an increase in lag time of conjugated diene formation. This was a very exciting finding as it suggests that in female runners, exercise actually results in a reduction in the susceptibility of LDL to oxidation (15) in trained runners. While these studies have involved half-marathon races, Liu et al reported an increase in oxidizability of LDL in male runners following a full marathon (12). The question of whether it was the increased distance that caused the increase in oxidizability, or the difference in gender, has yet to be explored. There is a good argument that these findings were gender related considering that the hormone estrogen, which occurs in higher levels in women, is also an antioxidant (66, 67).

Niess et al (68) investigated the response of inducible heme oxygenase, an antioxidant stress protein that is induced by reactive oxygen species, in endurance trained subjects following a half-marathon. They detected an increase in the cytoplasmic expression of heme oxygenase in the lymphocytes, granulocytes and monocytes. The observation that increases occurred immediately following exercise indicates that heme oxygenase was induced by reactive oxygen species generated during exercise. If the reactive oxygen species had been the result of muscle damage, a delayed response would have been observed. These researchers did not measure any parameters of oxidative stress such as lipid peroxidation or DNA damage markers and therefore it is speculation that oxidative stress occurred.

Sumida et al measured urinary 8-hydroxydeoxyguanosine (8-HOdG), a marker of in vivo oxidative DNA damage repair in 11 male long distance runners participating in a half-marathon run. They found no change in 8-HOdG 1-3 days following the run indicating that DNA damage was not present, but they could not rule out other types of oxidative damage because other markers were not measured.

In contrast to Sumida's study, Okamura and colleagues studied endurance trained subjects who ran 30 ± 6 km/day for eight days in addition to daily weight training. They reported increased levels of 8-HOdG levels in the urine of athletes, but no change in the 8-HOdG/gG ratios of lymphocytes (69). They concluded that repeated heavy exercise induces oxidative DNA damage as evidenced by increased excretion of urinary 8-hydroxydeoxyguanosine, but not lipid peroxidation (69).

Few studies have looked at longer distance running, but one study by Kanter et al (70), reported increases in MDA levels immediately following an 80 km race. Considering the variation in assays used to assess oxidative damage, it is difficult to draw a conclusion from a study that measured only one parameter of oxidative stress, but the results do imply that longer term exercise is associated with increased lipid peroxidation.

2.7. TYPES OF EXERCISE

Duration of exercise is likely to impact reactive oxygen species production. For example, no increases in malondialdehyde (MDA) levels, a marker of lipid peroxidation, were observed in athletes after either a 20 km run (17), or a 41 km marathon (14), but after an 80 km run Kanter et al (70) demonstrated a 77% increase in MDA levels. To complicate the interpretation of such results, the type of exercise also plays a role. Viinikka et al (71) found that TBARS, were unchanged after a 10-14 minute cycle ergometer test to volitional exhaustion. In

contrast, Lovlin et al (72) reported significant elevations in plasma MDA after intermittent high intensity concentric exercise on a cycle ergometer.

2.8. ANTIOXIDANT SUPPLEMENTATION

Hypothetically, antioxidant supplements should offer protection against exercise-induced oxidative stress, but this has not always been the observed result (53). Only a few studies have examined the effects of vitamin E supplementation on exercise induced lipid peroxidation (52-54). Vitamin E supplementation had no effect on urinary TBARS for 72 hours after a 45 minute downhill run (48) whereas vitamin E supplementation inhibited serum MDA formation after a cycle ergometer test to volitional exhaustion (52). Only one study has examined vitamin C alone (73); 2000 mg vitamin C had no effect on the exercise induced increase in serum conjugated diene concentration. The supplemented group did see a faster decrease in conjugated diene concentration than the placebo group after exercise. A small number of studies have examined the effects of combinations of antioxidant supplements on exercise induced oxidative stress (47, 50, 53), also yielding inconsistent results. There are a number of explanations for the inconsistent findings in these exercise studies including: differences in the modes, duration, and intensity of exercise as well as variation in the methodologies used to assess lipid peroxidation. In addition, there were large discrepancies in the types and amounts of antioxidant supplements provided, and in the duration of supplementation. A lack of consensus regarding the effectiveness of antioxidants to inhibit exercise-induced oxidative damage makes this a very attractive area for research (74).

2.9. ADAPTATIONS TO ENDURANCE TRAINING

Endurance exercise has been studied in depth and a number of physiological adaptations to training have been identified. Systemic adaptations include decreased utilization of muscle glycogen as a fuel source, "glycogen sparing" and therefore delayed time to fatigue (23). Enhanced ability to use fat as a fuel through increased free fatty acid transport and metabolism (β oxidation) (75) contributes to the glycogen sparing effect observed in endurance trained individuals. Although maximal oxygen consumption (VO_{2max}) can be increased with training, steady state oxygen consumption (VO_2) during submaximal work is not affected by endurance training (75). Most of the adaptations mentioned so far are primarily due to changes that occur at the level of the muscle. In the actively trained skeletal muscle, increases in the size and number of mitochondria (64) leads to increased mitochondrial respiratory control. A reduction in the production of lactic acid and thus hydrogen ion production occurs in association with the glycogen sparing effect. As the trained individual breaks down less glycogen, there is a reduced flux through the glycolytic pathway and therefore less lactic acid is produced as a byproduct (75). It is not lactic acid itself, but its associated hydrogen ion, that contributes to muscle fatigue primarily through its effects on cell pH (23). Increased capillary density in the trained muscle allows more nutrients to be delivered to the tissues and increased waste removal (75). The increase in capillary density also contributes to decreased blood flow through the active muscle allowing for increased oxygen delivery and extraction. Finally, changes occur in the isomer of lactate dehydrogenase enzyme, the enzyme responsible for the conversion of pyruvate to lactate (75). With training, this enzyme is converted to the cardiac isomer, resulting in a shift in the reaction from the direction of lactate and H^+ to pyruvate and uptake into the mitochondria (75).

There is growing evidence that another adaptation to endurance exercise is an upregulation in the antioxidant defense system. Elevated basal levels of the antioxidant enzymes superoxide dismutase, glutathione peroxidase, catalase (13) and inducible heme oxygenase (68) have been reported in endurance trained individuals compared to untrained controls. In addition, elevated basal and post-exercise plasma levels of antioxidant molecules such as vitamin E (47), vitamin C (16, 17) and uric acid (10, 12, 52) have been reported as a response to exercise. Some investigations have revealed that endurance trained individuals are also more likely to have increased free radical scavenging capacity (10, 12). Despite the number of studies supporting an increased antioxidant defense system, an equal number of studies have disputed this idea and thus more research on this point is warranted.

Finally, training appears to increase resistance to muscle damage, as evidenced by a reduction in the serum creatine kinase response following exercise, a marker of muscle damage, (64). Taken together, these adaptations to endurance exercise contribute to a delayed time to fatigue, a reduction in exercise-induced muscle damage and increased endurance capacity.

3. Assessment of Oxidative Stress in Athletes During Extreme Endurance Exercise Using Deuterium-labeled Vitamin E

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To be submitted to *Free Radical Biology & Medicine*,
The Official Journal of The Oxygen Society

3.1. ABSTRACT

To determine whether extreme endurance exercise induces lipid peroxidation, we studied 14 athletes (5 females: 9 males) during a 50 km ultramarathon (trial 1) and during a sedentary protocol (trial 2) one month later. At dinner the evening before the race (or sedentary trial), subjects consumed vitamin E labeled with stable isotopes (75 mg each d_3-*RRR* and d_6-*all rac*-α–tocopheryl acetates). Blood samples were taken at baseline, 30 minutes pre-race, mid-race, post-race, 1 h post-race, 24 h post-race, and at corresponding times for each individual during trial 2. During the sedentary day of trial 2, subjects consumed the same amounts of race day foods, including ergogenic aids, that they had consumed in trial 1; vitamin E intakes were 77 ± 40 mg, and vitamin C 406 ± 169 mg. All 14 subjects completed the race; average time to completion was 390 ± 67 minutes. Plasma F_2-isoprostanes (F_2-I), labeled and unlabeled α-tocopherol, and ascorbic acid (AA) were measured. F_2-I increased from 76 ± 24 pg/ml pg/ml at pre-race to 117.4 ± 38.0 pg/ml ($p<0.0008$) at mid-race to 130 ± 54 pg/ml ($p<0.0001$) at post-race, then returned to baseline at 24 hours post-race; F_2-I were unchanged during trial 2. Deuterated d_3 α-tocopherol (d_3 α-Toc) disappearance rates were faster ($2.8x10^{-4} \pm 0.5x10^{-4}$) during the race compared to the sedentary trial ($2.3x10^{-4} \pm 0.6x10^{-4}$; $p < 0.03$). Plasma AA *increased* from 75.2 ± 11.2 μM at pre-race to 157.7 ± 36.2 μM at race end ($p<0.0001$) and decreased to below baseline concentrations at 24 h post-race (40.4 ± 5.2 μM $p<0.0001$). AA levels also increased during trial 2. Despite increased plasma AA, F_2-I increased during exercise, but not during the sedentary period. Additionally, vitamin E disappeared faster during the run compared to the sedentary protocol. Thus, extreme endurance exercise results in the generation of lipid peroxidation and increased vitamin E utilization.

3.2. INTRODUCTION

Despite the many known health benefits of exercise, it has been hypothesized that endurance exercise causes oxidative stress based on the increased electron flow through the electron transport chain resulting from increased oxygen consumption associated with aerobic exercise. During the resting state, the human body produces reactive oxygen species (ROS), but at levels well within the capacity of the body's antioxidant defense system. During endurance exercise there is a 10 to 20-fold increase in whole body oxygen consumption (2), and oxygen uptake in the active skeletal muscle increases 100 to 200-fold (1, 3). This elevation in oxygen consumption may result in the production of ROS at rates that exceed the body's capacity to detoxify them (4), leading to the accumulation of excess ROS, which can in turn cause lipid peroxidation, as well as nucleic acid and protein damage (5). Specific sources of ROS during exercise include leakage of electrons from the mitochondrial electron transport chain (1), xanthine oxidase, damage to iron-containing proteins, and disruption of Ca^{2+} homeostasis, among others (26). Since vitamin E (α–tocopherol) is the antioxidant primarily responsible for scavenging peroxyl radicals (6), an increase in lipid peroxidation could deplete vitamin E stores. Davies et al (7) conducted one of the earliest studies addressing the issue of oxidative stress associated with exercise. They reported in male Long Evans rats that exercise increased lipid peroxidation, as measured using the TBARS assay. Since that time, studies have been carried out in humans to evaluate whether exercise increases oxidative stress; some studies have supported Davies' findings (8-13), while others have refuted them (14-17).

There is a great deal of controversy over whether or not oxidative stress and subsequent damage are truly associated with exercise. There are a number of

factors contributing to this lack of consensus. In the past, exercise studies have varied in the intensity, duration and mode of activity chosen for the study model. Additionally, variation in the fitness levels of subjects and assays used to assess oxidative damage contributed to the inconsistent findings. Therefore, we have used the most modern and reliable tools available, stable isotope labeled vitamin E and F_2-isoprostanes, to assess oxidative stress in endurance exercise.

Duration of exercise is likely to impact reactive oxygen species production. For example, no increases in MDA levels, were observed in athletes after either a 20 km run (17), or a 41 km marathon (14), but after an 80 km run Kanter et al (70) demonstrated a 77% increase in MDA levels; the present study used a 50 km run as the exercise model.

Previous methods used to assess lipid peroxidation have lacked sensitivity and/or specificity and are unreliable (56). This helps to explain why several similar studies involving trained male athletes running 21 km races, yielded conflicting post-race results: an elevation (10, 13) or no change (11, 17) in TBARS. Similarly, Case et al (15) reported an *increase* in lag time of conjugated diene formation in LDL obtained after a half-marathon, whereas Liu et al (12) reported a *decrease* after a full marathon. More puzzling, Marzatico et al (13) noted an increase in MDA levels after a half marathon, but no change in plasma conjugated dienes.

The present study utilizes the recently developed F_2-Isoprostanes assay to assess lipid peroxidation. F_2-Isoprostanes are a reliable measure of in vivo lipid peroxidation (57) with a sensitive lower limit of detection in the picogram range (58). They are specific end-products of the non-enzymatic free-radical catalyzed oxidation of arachidonic acid (59). F_2-Isoprostanes have biological activity of their own; they may act as potent vasoconstrictors and/or cause platelet aggregation (58).

Importantly, F_2-isoprostanes have been shown to be elevated in the plasma of subjects with known oxidative stress, such as smokers and patients suffering from diabetes, cystic fibrosis and Alzheimer's diseases (61-63).

α–Tocopherol acts to protect polyunsaturated fatty acids (PUFA) within phospholipids of biological membranes against lipid peroxidation (6). Although some researchers have looked at the effects of vitamin E supplementation on athletic performance/endurance (50, 51) and lipid peroxidation (52-54), none have used vitamin E biokinetics to evaluate oxidative stress. Previously, studies using deuterated α–tocopherols demonstrated that the various forms of vitamin E are absorbed and secreted similarly in chylomicrons, but that *RRR*- α–tocopherol is preferentially incorporated into VLDL in the liver as a result of the activity of the α–tocopherol transfer protein (α–TTP). Based on this mechanism, a mathematical model has been designed to determine the fractional disappearance rates of deuterated α–tocopherols (41). If exercise increases lipid peroxidation, increased amounts of vitamin E should be oxidized during its chain-breaking antioxidant role. To test this hypothesis, trained athletes were studied during a race and an equivalent sedentary period to evaluate whether exercise increases lipid peroxidation and the rate of plasma vitamin E disappearance.

3.3. METHODS

3.3.1. Subjects and Study Design

All experimental procedures were approved by the Institutional Review Board for the Protection of Human Subjects, Oregon State University. This study was designed to evaluate whether oxidative stress is generated during ultra-endurance exercise. The model used was a 50 km (32 mile) ultramarathon run that

takes place annually in Corvallis, OR. The ultramarathon consists of a trail run over rugged terrain, with a total elevation gain and loss of >12,000 ft. We studied 14 athletes (5 females: 9 males) during a 50 km ultramarathon (trial 1) and during a sedentary protocol (trial 2) one month later. The sedentary control protocol was identical to Trial 1, minus the exercise component. Subjects consumed a specified diet for 3 days prior to the race, on race day (or sedentary day) and for one additional day post-race (Table 1). At dinner the evening before the race (or sedentary trial), subjects consumed vitamin E labeled with stable isotopes (75 mg each d_3-*RRR* and d_6-*all rac*-α–tocopheryl acetates). Blood samples were taken at baseline (Day 1), 30 minutes pre-race (Day 4), mid-race, post-race, 1 hour post-race, 24 hours post-race (Day 5) and at corresponding times during trial 2. During trial 2, subjects consumed the same amounts of race day foods, including ergogenic aids that they had consumed ad libitum in trial 1; vitamin E intakes were 77 ± 401 mg, and vitamin C 406 ± 169 mg.

Table 1. Study Design

Trial 1 and Trial 2, Race and Sedentary Day, respectively

Day 1	Day 2	Day 3	Day 4	Day 5
Controlled Diet Baseline Draw	Controlled Diet	Controlled Diet Vitamin E Administration	Controlled Diet Pre-race Draw Mid-Race Draw Post-Race Draw Post-1 h Draw	Controlled Diet Post 24 h Draw

Volunteers were recruited from participants in the McDonald Forest Ultramarathon Race, an annual event carried out in Corvallis, Oregon. Approval to recruit subjects was given by the race promoter and an agreement was made to distribute the recruitment flier in the confirmation packet sent out to the race participants. Subjects were recruited from the pool of volunteers that responded to the flier. The pool was narrowed based on telephone questionnaires followed by in person interviews. The physical characteristics of subjects are presented in Table 2.

Table 2. Subject Characteristics

All Subjects	Weight (kg)	Age (years)	Height (cm)	Training (mile/week)
Mean	70.7	43	173.2	33
Std Dev	11.7	10	10.9	12
Females				
Mean	58.7	41	161.3	32
Std Dev	4.9	10	3.592	6
Males				
Mean	77.4	44	179.8	33
Std Dev	8.5	11	7.046	15

Mean age was 43 ± 10 yrs, weight was 57.5 ± 3.5 kg for females and 77.4 ± 8.5 kg for males; they averaged 33 ± 12 miles training/week (Table 1). Subjects were selected on the basis of non-smoking status, age 21-60 yrs, stable body weight, and maintenance of regular exercise patterns. Subjects were excluded if they were antioxidant supplement users (vitamin C, vitamin E, selenium or carotenoids) and were unwilling to discontinue supplements for 1 month prior to the study. Other exclusion criteria included vegetarian or restrictive dietary requirements, or a history of diabetes or uremia.

3.3.1.1. Exercise Training:

For the two weeks prior to the ultramarathon, subjects recorded on a daily basis the type, duration and intensity of exercise performed each day. For the sedentary trial, subjects maintained the same activity level for 3 weeks prior to the test day. Again, the subjects recorded on a daily basis the amount, type, and intensity of exercise performed each day. During the "sedentary" day (equivalent to race day) subjects were asked to avoid physical activity of any kind.

3.3.1.2. Energy Expenditure

To estimate caloric expenditure during the race, the Metabolic Equivalents equation was used. This equation is based on the relationship between the relative O_2 requirement ($ml \cdot kg^{-1} \cdot min^{-1}$) and running speed, which is a straight line, making the calculation of energy cost easy and reasonably precise (75). One Metabolic Equivalent (MET) is equal to resting oxygen consumption (VO_2) or ~3.5 $ml \cdot kg^{-1} \cdot min^{-1}$, thus the energy cost of exercise can be described in multiples of resting oxygen consumption (75).

Energy Expenditure (kcal/min)

= 0.0175 kcal $\cdot$ kg^{-1} $\cdot$ min^{-1} $\cdot$ MET^{-1} X METS (based on run speed) X body wt (kg)

Example: Energy Expenditure

= 0.0175 kcal $\cdot$ $kg^{-1} \cdot min^{-1} \cdot$ MET^{-1} X 10 METS (based on run speed) X 70 kg

= 12.25 kcal/min

*For a running pace of 10 min$\cdot$ $mile^{-1}$, METS activity = 10 METS (75)

3.3.1.3. Diet

Subjects consumed a specified diet for 5 days (3 days prior to the race, race day, and 1 day post-race) in trial 1 and 5 days (3 days prior to the sedentary protocol, day of the sedentary protocol, and 1 day post-sedentary protocol) in trial 2. All foods were prepared and provided to the participants in the Metabolic Feeding Unit in the Human Nutrition Research Laboratory. The daily diet provided ~2800 Kcal for the females and ~3300 Kcal for the males and consisted of approximately 12-15% protein, 55-65% carbohydrate, and 25-30% fat. During the race, selected foods and beverages were available for consumption at checkpoints along the racecourse. Except for up to 500 ml of water, the subjects were not allowed to consume any food or beverages for one hour after the race. The types, amounts and times of consumption were recorded for all race day foods. During the sedentary trial, subjects were provided with the same forms and amounts of foods as they had consumed on race day and were instructed to consume them at the corresponding times as during the race trial.

Nutrient composition of the specified diets was determined using the Food Processor Program (ESHA Research, Salem, OR).

3.3.1.4. Vitamin E Supplementation: Deuterated Tocopherols

12 hours prior to the race (or sedentary protocol) all subjects consumed a single gelatin capsule containing 150 mg deuterium labeled vitamin E (75 mg each *RRR*-α–[5(C^2H_3)]-and *all rac* α– [5,7($C^2H_3)_2$]-tocopheryl acetates (d_3 *RRR* α– and d_6 *all rac*- α– tocopheryl acetates, respectively) with dinner. Capsules were provided by NSVEA (Natural Source Vitamin E Association, Washington, D.C.) who attests to their purity for consumption by humans.

Some subjects were non-compliant; 3 of the 14 subjects did not consume the deuterium labeled vitamin E capsule as evidenced by the lack of a plasma response of deuterated α-tocopherol level, or in the case of one subject, a response only in Trial 2. Although the researcher monitored each subject as they consumed the capsules, this was not 100% effective and clearly a more rigorous method will be called for in future.

Due to non-compliance, data for antioxidants is reported for all 14 subjects, but data for vitamin E is only reported for 11 subjects.

3.3.1.5. Blood Draws

Blood was drawn from the antecubital vein by a trained phlebotomist. Blood draws occurred 3 days prior to the race (fasting), 30 minutes prior to the race, during the race at kilometer 27, immediately after race completion, 60 minutes following the race, and again 24 hours post-race (Table 1). 19 ml was drawn each time, except for the 5^{th} day draw, which was only 5 ml, for a total of 6 blood draws per trial equaling 100 ml. Only a portion of the blood drawn was used for this study, the remainder was used for a study of vitamin B_6 metabolism to be reported elsewhere (76). Blood samples during the sedentary trial were timed to occur at equivalent time points as during the race.

3.3.1.6. Plasma Samples

Blood was drawn into two 5 cc purple-top Vacutainer tubes (containing 1 mg/ml EDTA). An aliquot was taken immediately to measure hematocrit and hemoglobin in order to correct samples for plasma volume changes. Plasma was then obtained by centrifugation at 2500 x *g* for ten minutes, transferred into

appropriate storage tubes, flash frozen in liquid nitrogen and stored at -80° C. All samples were analyzed within 6 months of collection.

For vitamin C analysis, plasma samples were taken from blood collected in 5 cc green-top Vacutainer tubes (containing 143 USP sodium heparin). 50 μL of the freshly drawn plasma was mixed with an equal volume of chilled 5% (wt/vol) metaphosphoric acid in 1 mM diethylenetriamine pentaacetic acid (made fresh daily) and centrifuged to remove the precipitated proteins. A portion of the supernatant was frozen at -80° C until day of analysis, which was within 1 month of sample collection.

3.3.1.7. Plasma Volume Changes

Plasma volume changes were calculated from hematocrit and hemoglobin using the methods of Dill and Costill (77). All analyses were calculated with and without plasma volume changes. Adjusting for changes in plasma volume had very little impact on significant differences and all parameters remained significant except those specified otherwise in the results section. Since some parameters, such as F_2-isoprostanes have biological activity, results are reported without adjusting for changes in plasma volume.

3.3.2. Sample Analyses

3.3.2.1. Ascorbic Acid and Uric Acid

Ascorbate and urate were determined by paired-ion reversed-phase HPLC coupled with electro-chemical detection using a modification of the method described by Kutnink et al (78). Upon analysis, acidified frozen supernatant, described above, was defrosted and 10 μL was mixed with 10 μL 1 mM DTPA, 74 μL ascorbic acid eluant (see below) and 6 μL of 2.58 M K_2HPO_4 buffer (pH 9.8).

20 μL of the mixture was immediately injected with an ESA model 540 auto-sampler (Chelmsford, MA) and chromatographed on an LC-8 column [15 cm x 4.6 mm (i.d.)] (Supelco). The eluant was delivered at a flow rate of 1.0 mL/min, consisting of 40 mM sodium acetate, 0.51 mM DTPA, 1.5 mM dodecyltriethylammonium-phosphate, and 7.5% (vol/vol) methanol, adjusted to pH 4.75 with glacial acetic acid. The eluate was analyzed with an LC 4B amperometric electrochemical detector equipped with a glassy-carbon working electrode and a Ag/AgCl reference electrode (Bioanalytical Systems, West Lafeyette, IN), with an applied potential of +0.6 V, and a sensitivity setting of 500 nA for both urate and ascorbate. A set of ascorbate and urate standards with concentrations from 1.2 to 5 μM, and 7.2 to 30 μM, respectively (in 1 mM DTPA, in PBS) was run before and after each of the sample runs.

3.3.2.2. Vitamin E

d_0-, d_3-, and d_6-α–TAc standards were saponified using alcoholic KOH, as described previously (6). Briefly, known amounts of each of the tocopheryl acetates were dissolved in 2 ml ethanol (1% ascorbic acid, w:v), 0.3 ml saturated KOH, and 1 ml water for 30 min at 70°C. On cooling, 1 mL H_20 (1% ascorbic acid, w:v), and BHT (1mg/mL ethanol) were added. After hexane extraction, and nitrogen evaporation, the fractions were re-suspended in 1 ml MeOH:EtOH (1:1). Concentrations of free tocopherols were determined by UV absorption using a Beckman (Fullerton, CA) DU Series 600 spectrophotometer at 292 nm.

Tocopherols were extracted with hexane from human plasma (79) that had been saponified. A known aliquot of the supernatant was taken, internal standard (d_9 α-tocopherol) added, the hexane dried under nitrogen and the residue re-

suspended in 100 µl ethanol:methanol 1:1. All samples were stored at –20° C overnight before analysis.

For LC-MS/MS analysis, the method of Lauridsen et al (80) was followed. The high performance liquid chromatography (HPLC) system used consisted of a Shimadzu LC-10ADvp pump, a Shimadzu SIL-10ADvp auto injector with a 50 µl sample loop, a Shimadzu DGU-14A degasser, and a 7.5 cm x 4.6 mm, 3 µm particle Supercosil™ LC-18 column (Supelco, Bellfonte, PA). The mobile phase was 100% methanol, with a total run time of 4 min.

All samples were analyzed using a Perkin Elmer Sciex API III+ triple-quadrupole mass spectrometer (Perkin Elemer -Sciex Instruments, Thornhill, Ontario, Canada) using the atmospheric pressure chemical ionization (APCI) source. The corona discharge electrode was set to 5000 V and the probe temperature was set to 500°C. The curtain gas (nitrogen) was set to 0.6 L/min, the nebulizer gas (air) at 80 npsi, and the auxiliary gas (air) at 1 L/min. The orifice plate voltage was +55 V. MS/MS experiments were carried out with argon-nitrogen (9:1, Airco, Vancouver, WA, USA) as target gas at a thickness of 1.8×10^{14} molecules/cm^2, and the collision energy was adjusted to 25 V.

Quantitative MS/MS data were obtained by multiple reaction monitoring (MRM) of d_0, d_3, d_6, and d_9 in a 0-4 min period using RAD version 2.6 software (Ontario, Canada) as follows. d_0-α–tocopherol, *m/z* 430.4→165.2, d_3-α–tocopherol, *m/z* 433.4→168.2, d_6-α–tocopherol, *m/z* 436.4→171.2, and d_9-α–tocopherol, *m/z* 439.4→174.2 (Fig. 2). The dwell time for each pair of parent-product ions was 200 ms, allowing 1.67 scans per second. Peak areas of the above tocopherols were determined with the McQuan version 1.5 software (Ontario,

Canada), which allowed for quantitation of d_0, d_3, and d_6-α-tocopherols based on a d_9 internal standard.

The rate of disappearance of labeled vitamin E from the plasma was determined by fitting an exponential equation to each individual's time versus d_3/cholesterol ratios for each of the two protocols.

3.3.2.3. Plasma F_2-Isoprostanes

Plasma samples that had been snap frozen in liquid nitrogen were stored at -80° C until time of analysis. Free F_2-isoprostanes were measured in plasma using the Cayman 8-Isoprostane EIA Kit (Cayman Chemical, Ann Arbor, MI). For quantification of free F_2-isoprostanes, 1 ml plasma (immediately upon thawing) was added to 9 ml ultra-pure Milli-Q water, pH 3.0 containing 1000 cpm ^{3}H-$PGF_{2\alpha}$ (Amersham TRK 464). The mixture was then acidified to pH 3.0 by the addition of ~35 µl concentrated hypochlorous acid. Samples were purified on C18 SPE Cartridges (Sep-Pak, Waters) that had been preconditioned with 10 ml methanol, followed by 10 ml Milli-Q water, pH 3.0. Once loaded, the sample was washed with 10 ml Milli-Q water, pH 3.0, followed by 10 ml heptane. F_2-isoprostanes were eluted with 10 ml ethyl acetate/heptane (1:1). Further purification was accomplished by drying the eluate with anhydrous sodium sulfate and then loading the eluted sample onto Silica Cartridges (Waters) that had been activated with 5 ml methanol followed by 5 ml ethyl acetate. F_2-isoprostanes were then eluted with 5 ml ethyl acetate /methanol (1:1). The tubes were capped and stored at -80° C until time of the ELISA. Eluted samples were dried under nitrogen in a 37° C water bath. They were then resolved with 0.5 ml EIA buffer and vortexed for 15 seconds. 250 µl of sample was counted in a Liquid Scintillation Counter to determine percent recovery. The remaining sample was loaded onto an ELISA plate (Cayman Kit) in

quadruplicate wells. The plate was incubated overnight (10-12 hours) at room temperature. It was then developed with Ellman's Reagent and read with a Molecular Devices Thermomax Microplate Reader; wavelength 405 nm. Data were analyzed according to kit instructions.

3.3.2.4. Other Assays

Plasma triglycerides and cholesterol were measured by standard clinical assays (Sigma kit).

3.3.3. Statistical Analyses

Repeated measures ANOVA for a crossover design was used to analyze all data. Post-hoc analysis for significant differences: Fishers PLSD. An unpaired t-test was used to test for differences between the genders. Since subjects acted as their own controls, it was necessary to make a large number of statistical comparisons. Therefore, a more conservative approach was used; $p < 0.03$ cut-off for significance. Statistics were calculated using the Stat-view® program (SAS Institute Inc., Cary, NC).

3.4. RESULTS

3.4.1. Subject Characteristics

All 14 subjects (5 females: 9 males) completed a 50 km ultramarathon race; average run time was 6.5 ± 1.1 hours. An unpaired t-test revealed no significant differences between the males and females other than weight, height and energy expenditure.

We used the metabolic equivalents equation (METS) to estimate energy expenditure because it takes into account run time, average run pace and body

weight for each individual. In order to allow for differences in energy expenditure between the genders, the daily diets were adjusted so that caloric intake was approximately 500 kcal higher for the males than the females; subjects were allowed to consume specified foods ad libitum during the race.

For simplicity, we have grouped the data for both genders and from this point forward all results will be reported for the group as a whole. During the race, subjects consumed 2042 ± 960 Kcal, 486 ± 235 g carbohydrate, 11 ± 15 g fat, 406 ± 169 mg vitamin C and 77 ± 40 mg vitamin E (Table 3). The vitamin E and vitamin C supplements were consumed in the form of ergogenic aids such as carbohydrate pastes and drinks. It is a common practice during endurance exercise for athletes to consume carbohydrate fuels in order to maintain plasma glucose levels and thus delay time to fatigue (23).

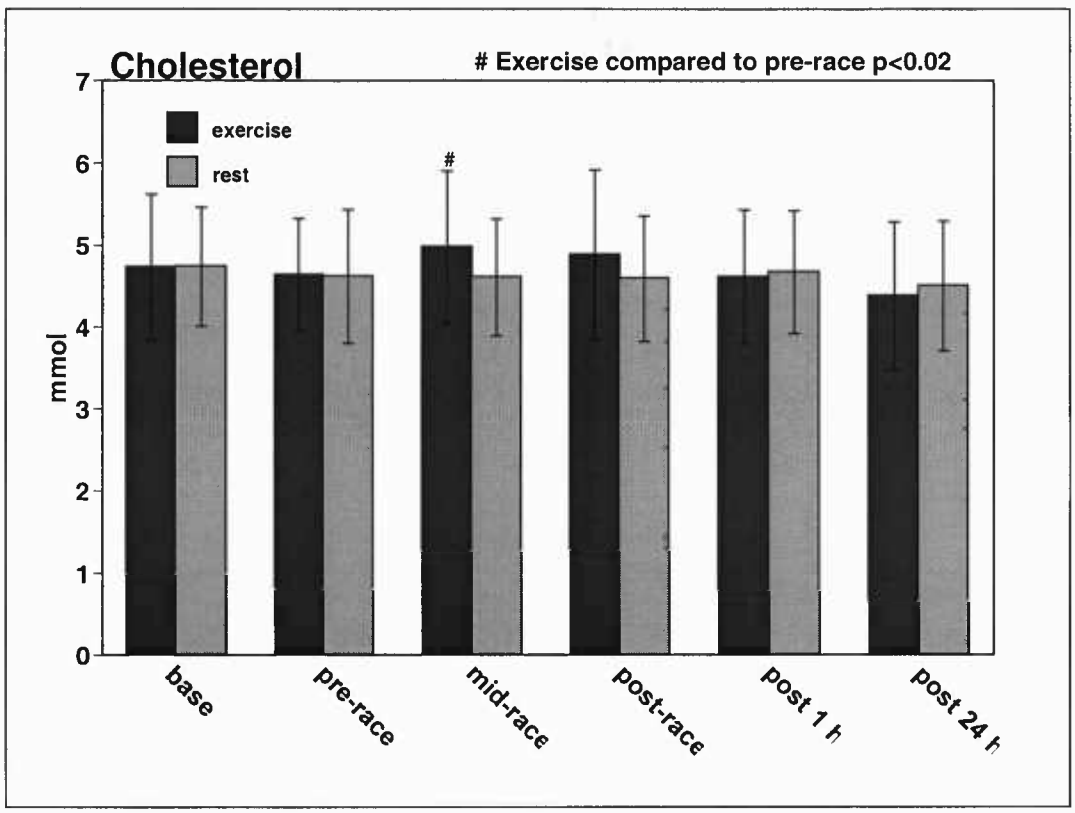
Table 3. Race Day Results

	Run Time	Rate	METS	EE	Intake	CHO	Vit C	Vit E	Fat
All Subjects	(min)	(min/mile)		(Kcal)	(Kcal)	(g)	(mg)	(mg)	(g)
Mean	390	12.6	7.4	3389	2042	486	406	77	11
± Std Dev	67	2.2	2.2	766	960	235	169	40	15
Females									
Mean	404	13.0	6.9	2788	1534	352	317	66	12
± Std Dev	52	1.7	1.7	462	904	200	170	32	20
Males									
Mean	382	12.3	7.7	3723	2325	560	455	84	11
± Std Dev	76	2.4	2.5	703	916	228	157	44	13

3.4.2. Serum Lipids

Vitamin E, a fat-soluble molecule, is absorbed and transported with lipids, therefore serum lipids were measured at all time points in all individuals. During

Figure 5. Plasma Cholesterol

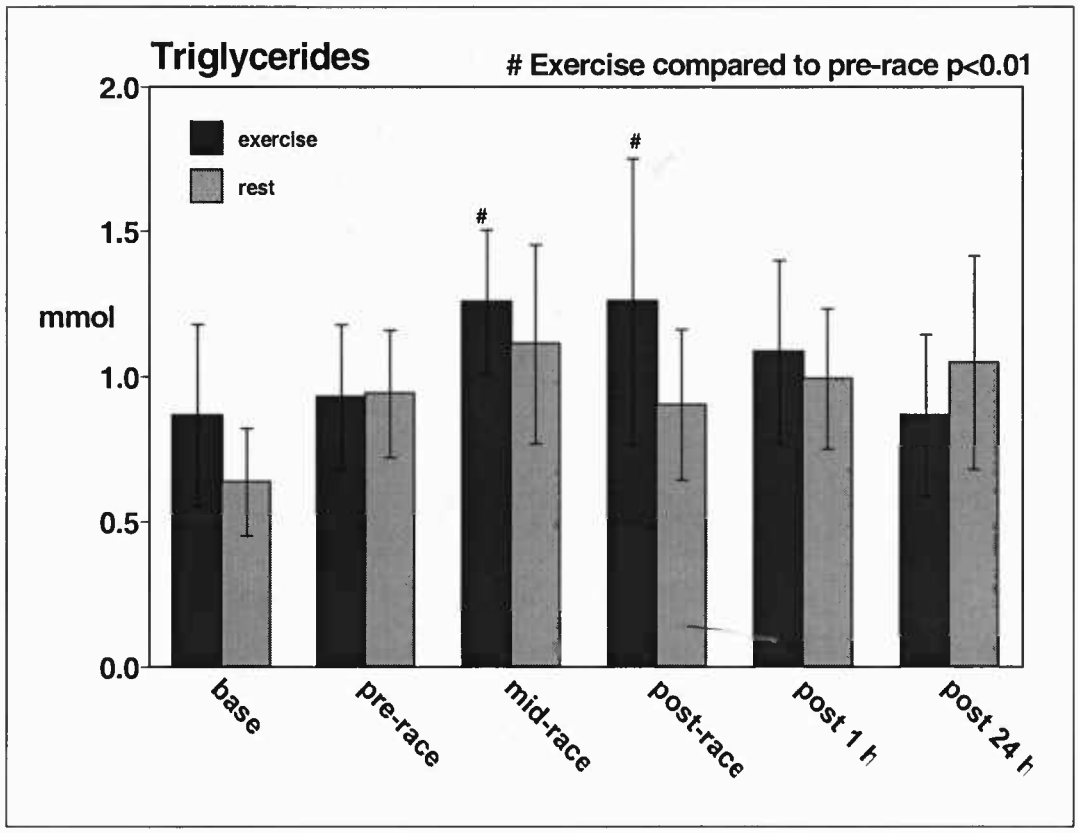


the race trial, plasma cholesterol concentration increased from 4.6 ± 0.7 μM 30 minutes prior to the race to 5.0 ± 0.9 μM by mid-race ($p<0.02$), but levels were not different from pre-race at any other time point (Figure 5). Plasma cholesterol concentration changes during the sedentary trial as well as during the race trial compared to the sedentary trial, were not statistically different.

During the race trial, plasma triglycerides increased from 0.93 ± 0.3 mmol/L 30 minutes prior to the race to 1.3 ± 0.2 mmol/L by mid-race ($p<0.01$) and 1.3 ± 0.5 mmol/L at post-race ($p<0.01$) (Figure 6). By 1 hour post-race, plasma levels had returned to near baseline levels (1.1 ± 0.3 mmol/L) and were returned to baseline by 24 hours post-race (0.9 ± 0.3 mmol/L). Similar to cholesterol, plasma triglycerides did not change during the sedentary trial. Plasma triglycerides were

not significantly different in the race trial compared to the sedentary trial at any time point.

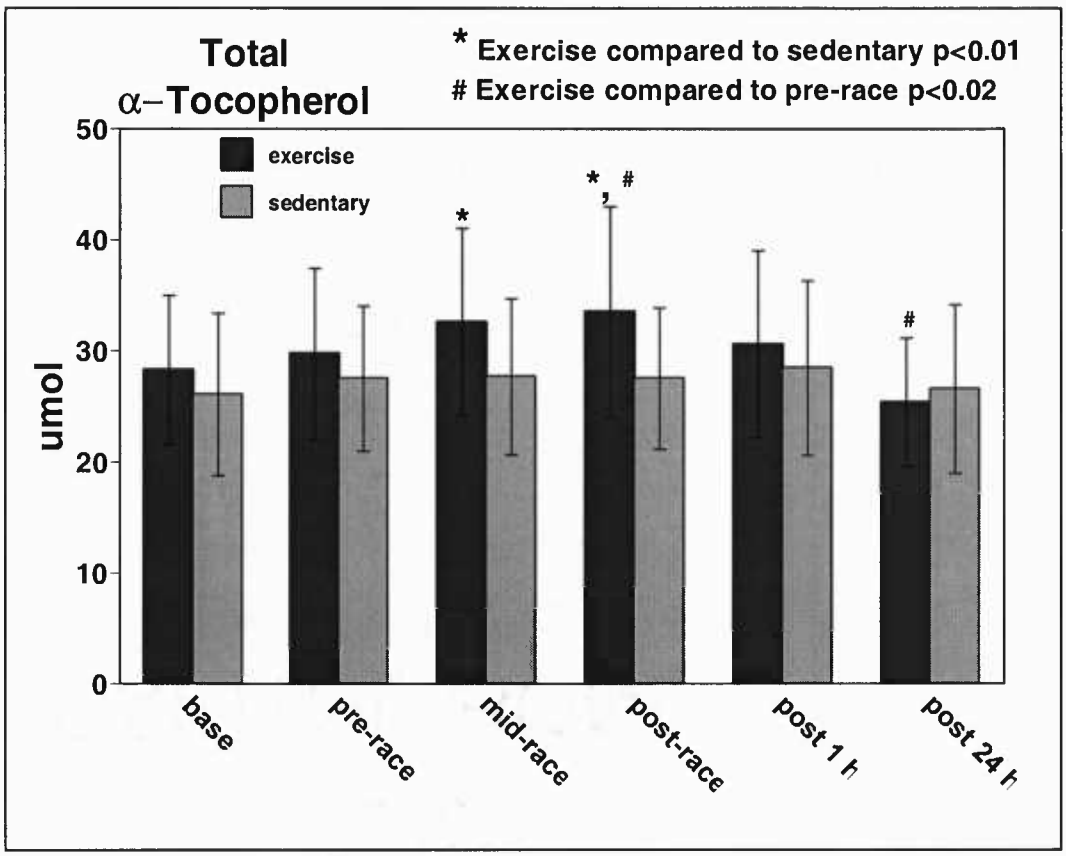
Figure 6. Triglycerides



3.4.3. Labeled and Unlabeled α-Tocopherol

During exercise induced oxidative stress and subsequent lipid peroxidation, we expected to see changes in the concentration of vitamin E because it is a potent peroxyl scavenger. However, during the race trial, total α-tocopherol in the plasma increased from 29.7 ± 7.7 μM 30 minutes prior to the race to 33.5 ± 9.5 μM at post-race ($p<0.02$) (Figure 7). Other studies involving extreme endurance exercise have also reported an increase in plasma α-tocopherol levels post exercise (47, 69)

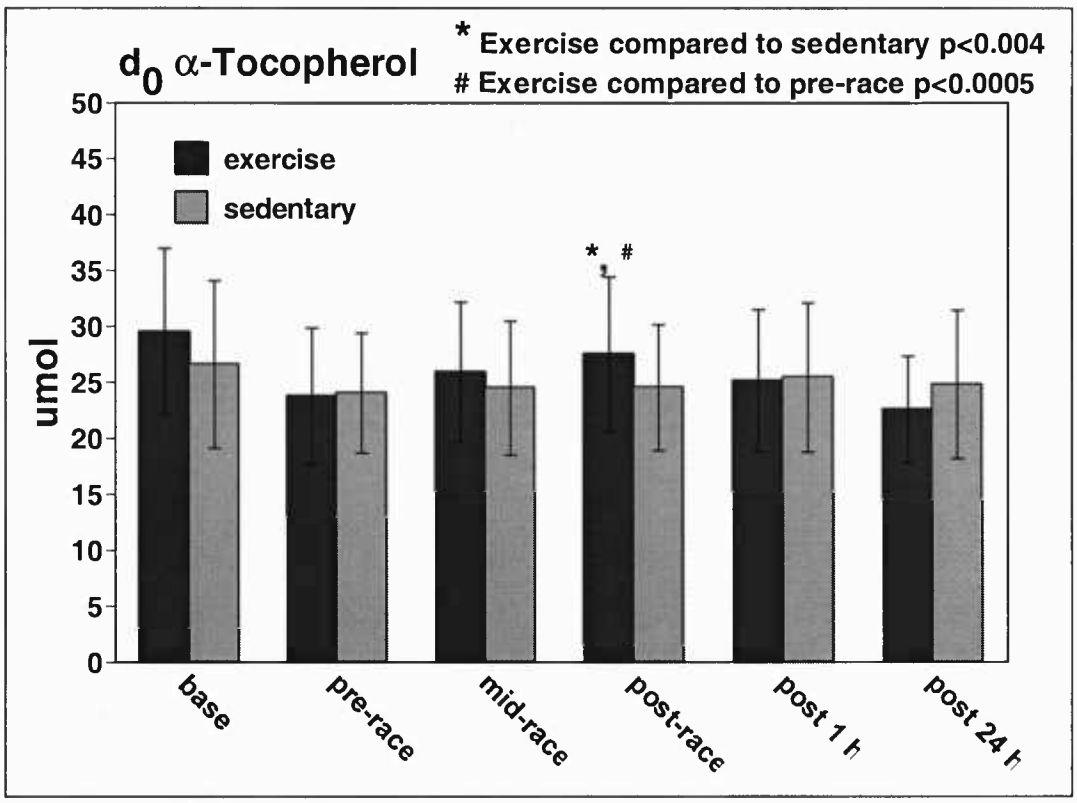
Figure 7. Total α-Tocopherol



whereas studies of shorter duration reported no change in vitamin E levels in response to exercise (16, 17, 48). Total α-tocopherol had returned to near pre-race levels 60 minutes post-race (30.5 ± 8.5 μM) but dropped to below pre-race levels (25.3 ± 5.8 μM) by 24 hours post-race ($p<0.007$). Plasma total α-tocopherol levels did not change during the sedentary trial. In the race trial compared to the sedentary trial, levels were elevated at mid-race ($p<0.01$) and post-race ($p<0.0003$) (Figure 7), indicating that the changes observed during the race trial were a response to exercise not the vitamin E in the ergogenic aids.

d_0 α-Tocopherol (unlabeled α-tocopherol),was also measured. During the ultramarathon run, plasma d_0 α-tocopherol increased from 23.7 ± 6.1 μM 30 minutes prior to the race to 27.4 ± 6.9 μM ($p<0.0005$) at post-race (Figure 8). In

Figure 8. d_0 α-Tocopherol



contrast, d_0 α-tocopherol remained constant during the sedentary trial. d_0 α-Tocopherol levels were elevated at post-race ($p<0.003$) in the race trial compared to the sedentary trial (Figure 8). Since d_0 α-tocopherol concentrations increased during the exercise trial but not the sedentary trial, the increase is likely a response to the exercise, rather than the vitamin E in the ergogenic aids.

We used d_3 labeled α-tocopherol, consumed 12 hours prior to exercise, to track the kinetics of vitamin E. During the ultramarathon run, plasma d_3 α-tocopherol did not change. However, at 24 hours post-race, d_3 labeled α-tocopherol decreased from 4.1 ± 2.2 μM 30 minutes prior to the race to 2.3 ± 1.2 μM ($p<0.0001$) (Figure 8).

This time frame is consistent with the disappearance of d_3 labeled α-tocopherol leaving the plasma reported by Traber et al (41). A similar pattern was observed during the sedentary trial; plasma d_3 α-tocopherol did not change significantly until 24 hours post-trial. At 24 hours post-trial, d_3 labeled α-tocopherol decreased from 2.7 ± 1.5 μM 30 minutes prior to the race to 1.6 ± 0.9 μM ($p<0.0001$) (Figure 9).

At baseline, plasma d_3 α-tocopherol levels were slightly but significantly higher at the sedentary compared to the race baseline time point ($p< 0.01$), Figure 9, but the values were so small as to be physiologically inconsequential. Thus, one month was sufficiently long for the labeled tocopherol to leave the system.

Figure 9. d_3 α-Tocopherol

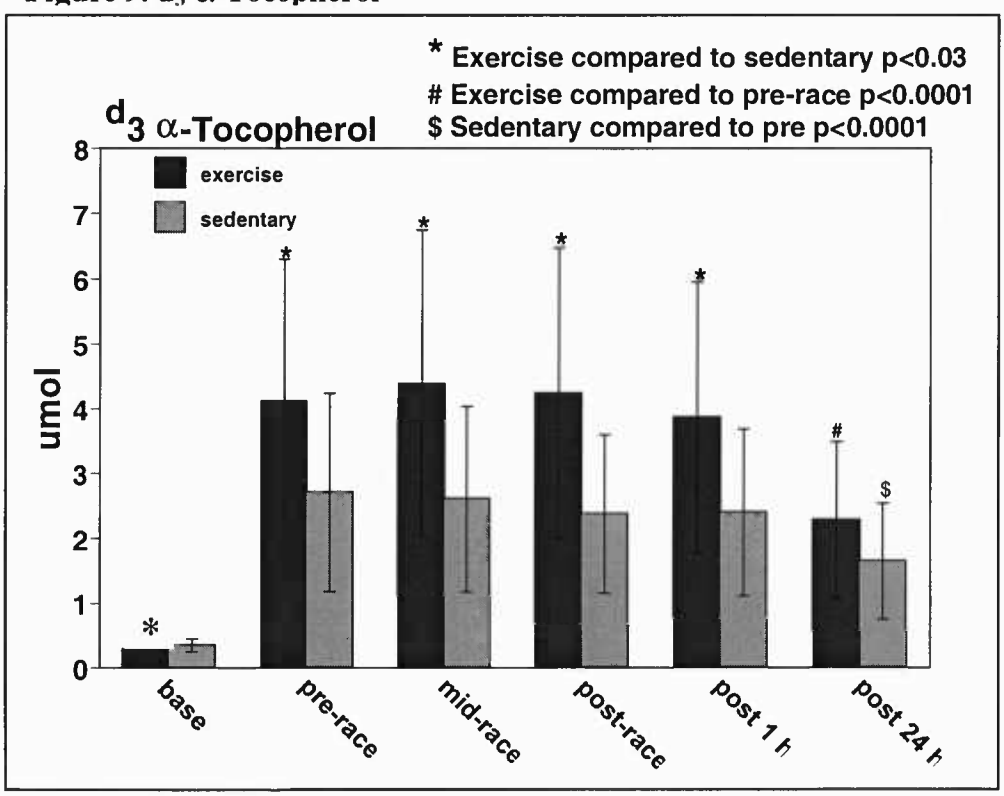
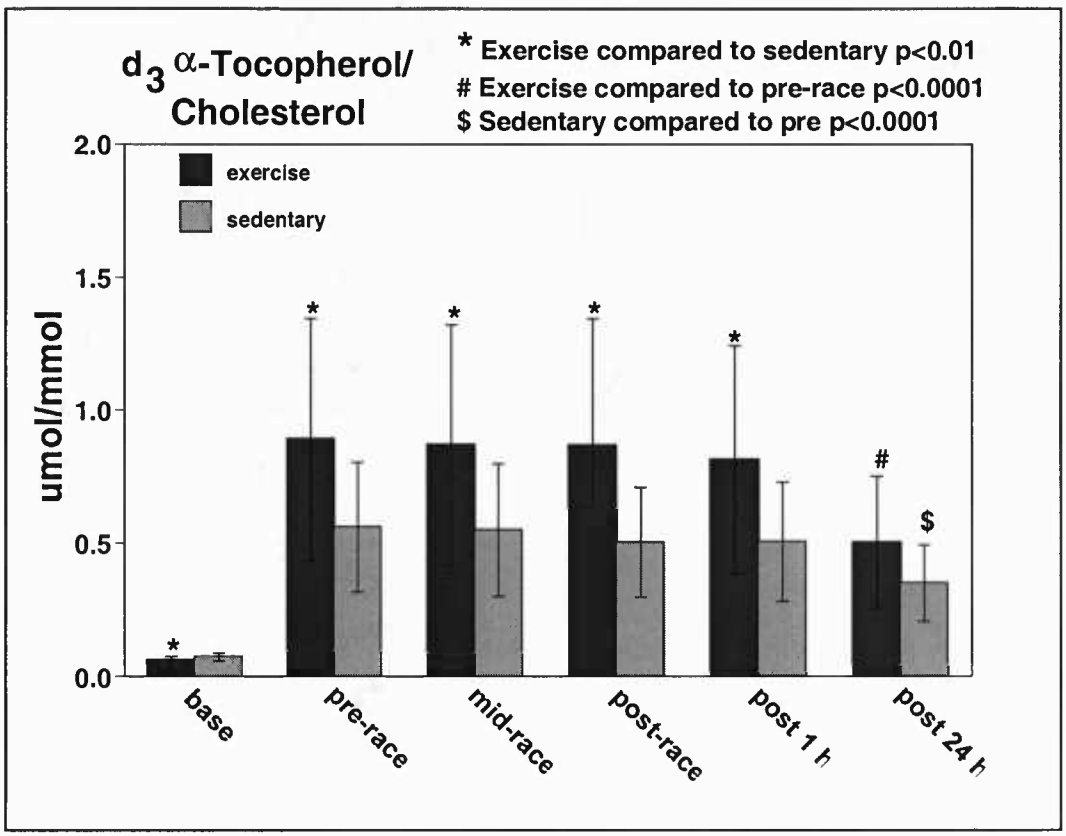


Figure 10. d_3 α-Tocopherol/Cholesterol vs. Time



Plasma d_3 α-tocopherol levels were elevated at all other time points during the race trial compared to the sedentary trial ($p<0.03$) except 24 hours post-race which was not different (Figure 9). This was an unanticipated result as we would have expected the pre-trial values to be identical in both trials as this time point was positioned 14 hours after consumption of the labeled vitamin E capsule. d_6 α-Tocopherol followed an identical pattern as d_3 α-tocopherol throughout both trials.

In order to account for changes in lipoprotein secretion and normalize the data for inter-subject variability in cholesterol levels, we calculated d_3 labeled α-tocopherol per cholesterol (d_3/cholesterol) (Figure 10).

The average d_3/cholesterol did not change during the race trial, not even at the 24 hour time point as observed for d_3 labeled α-tocopherol alone. Plasma d_3 α-tocopherol/cholesterol in the sedentary trial did not change throughout the trial. d_3 α-Tocopherol/cholesterol levels were elevated at 30 minutes prior to the race ($p<0.01$) and post-race ($p<0.006$) in the race trial compared to the sedentary trial, similar to the pattern observed for total α-tocopherol.

In order to determine vitamin E kinetics, we calculated the exponential rate of d_3/cholesterol disappearance for each subject based on their individual race time and blood draw time schedule. The d_3/cholesterol ratios for all time points for both trials in a representative subject are shown in Figure 11.

Figure 11. d_3 α-tocopherol/Cholesterol Disappearance

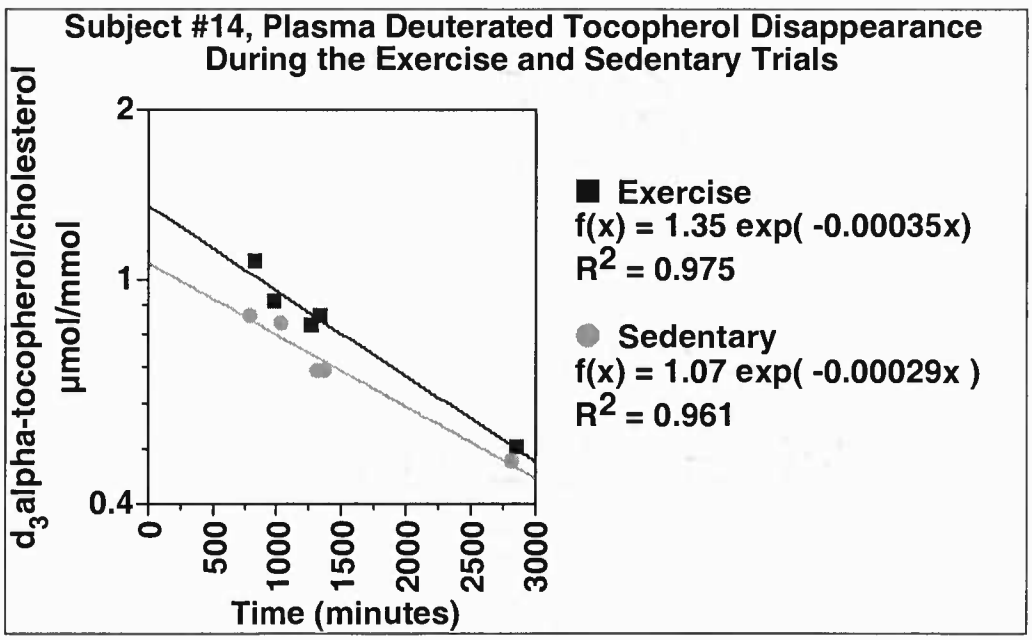
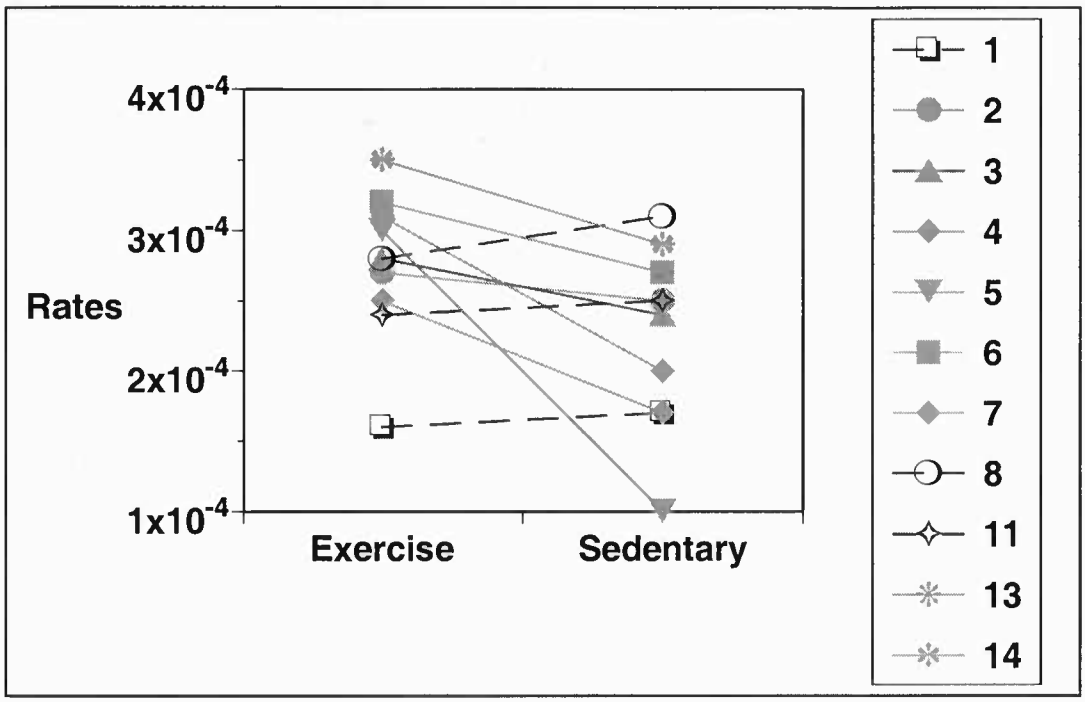


Figure 12. Individual d_3 α-tocopherol/Cholesterol Disappearance Rates

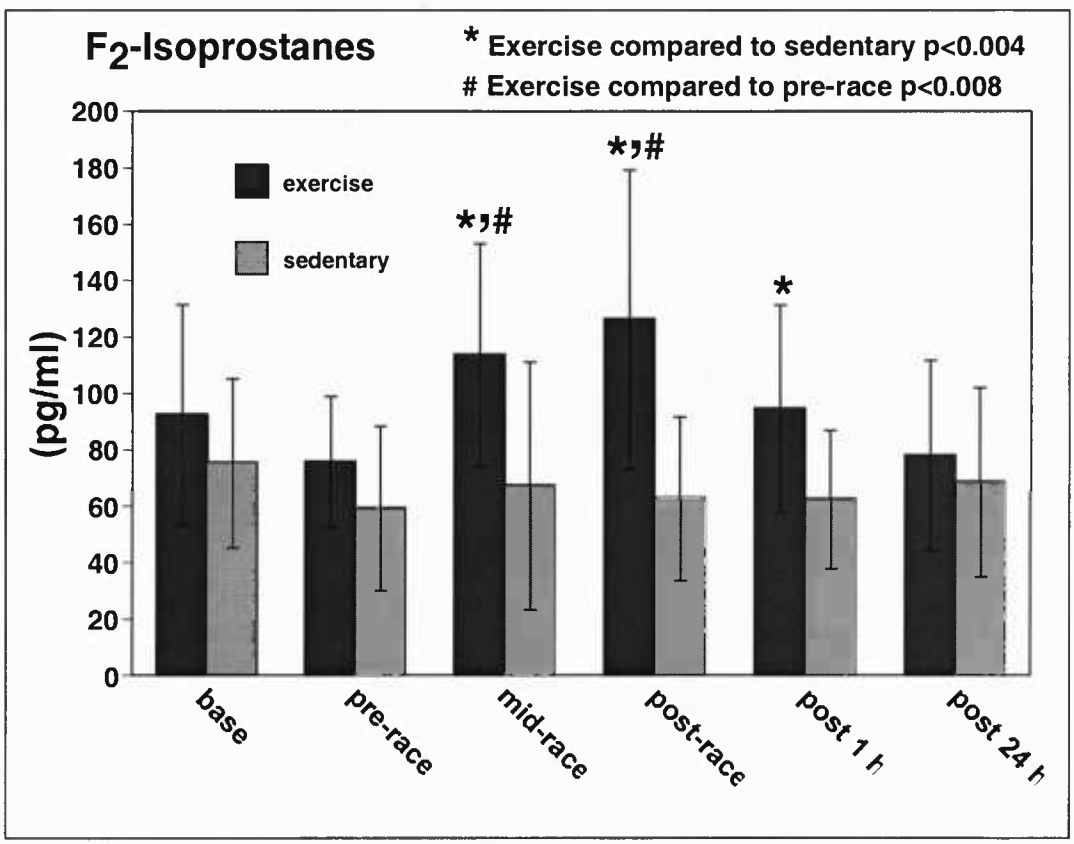


Each of the individual rates for the race and the sedentary protocols for all subjects, calculated based on their individual plasma d_3/cholesterol ratios, are shown in Figure 12. The disappearance rates were faster for eight of the eleven subjects during exercise compared to the sedentary protocol. The average plasma disappearance rate of d_3/cholesterol was faster ($2.8x10^{-4} \pm 0.5x10^{-4}$) during the race compared to the sedentary trial ($2.3x10^{-4} \pm 0.6x10^{-4}$; $p < 0.03$). This indicates that plasma disappearance of vitamin E was faster during the race compared to the sedentary trial, a novel finding.

3.4.4. Isoprostanes

Since vitamin E is a peroxyl radical scavenger, and vitamin E levels changed in response to exercise, it was of interest to assess lipid peroxidation. F_2-isoprostanes shifted dramatically in response to exercise, pointing to an increase in oxidative stress.

Figure 13. F_2-isoprostanes

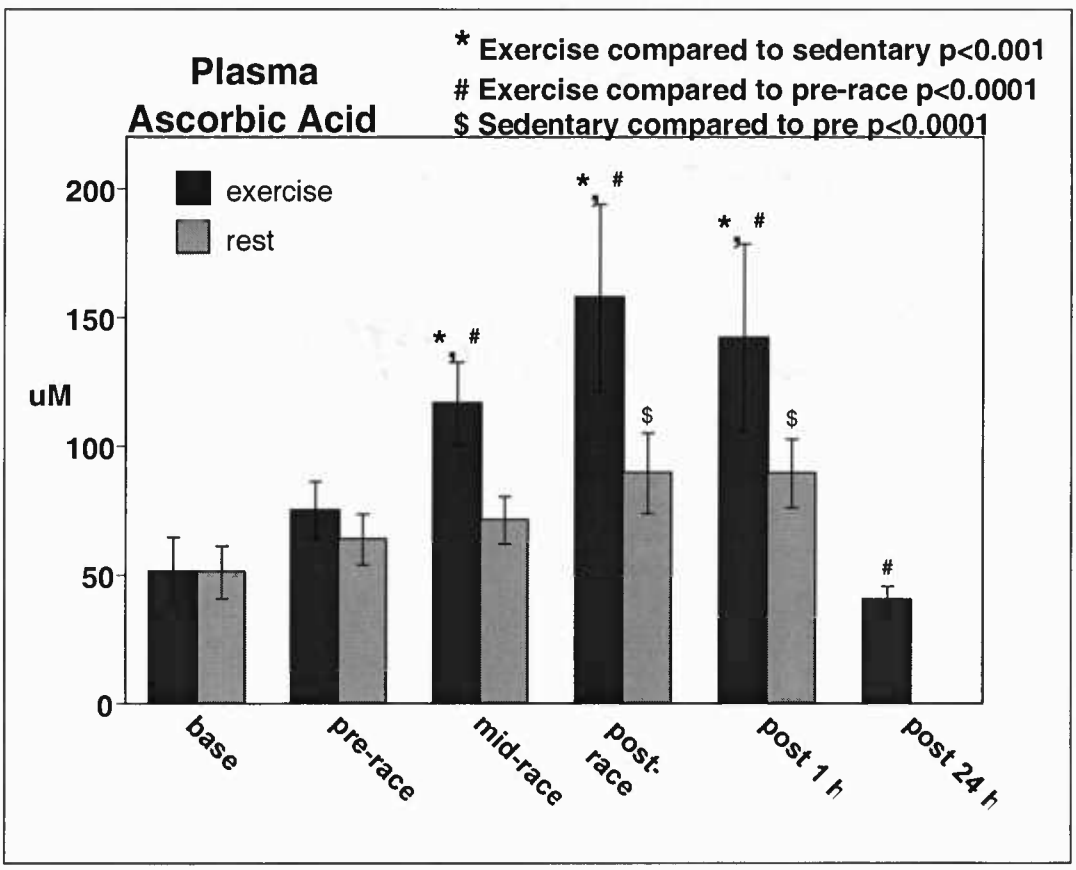


F_2-isoprostane levels increased from 76 ± 24 pg/ml 30 minutes prior to the race to 117 ± 38 pg/ml by mid-race ($p<0.0008$) and 130 ± 54 pg/ml at race end ($p<0.0001$) (Figure 13). F_2-isoprostane levels returned to near pre-race levels 24 hours post-race (79 ± 35 pg/ml). These differences remained significant after

adjusting values for changes in plasma volume. Importantly, plasma levels of F_2-isoprostanes did not change during the sedentary trial.

F_2-isoprostane levels were elevated at mid-race ($p<0.004$), post-race ($p<0.0007$) and 60 minutes post-race ($p<0.004$) during the race trial compared to the sedentary trial. The differences at mid-race ($p<0.03$) and post-race ($p<0.002$) remained significant after adjusting data for changes in plasma volume. This data offers strong evidence that lipid peroxidation resulted from increased oxidative stress in response to exercise.

Figure 14. Ascorbic Acid



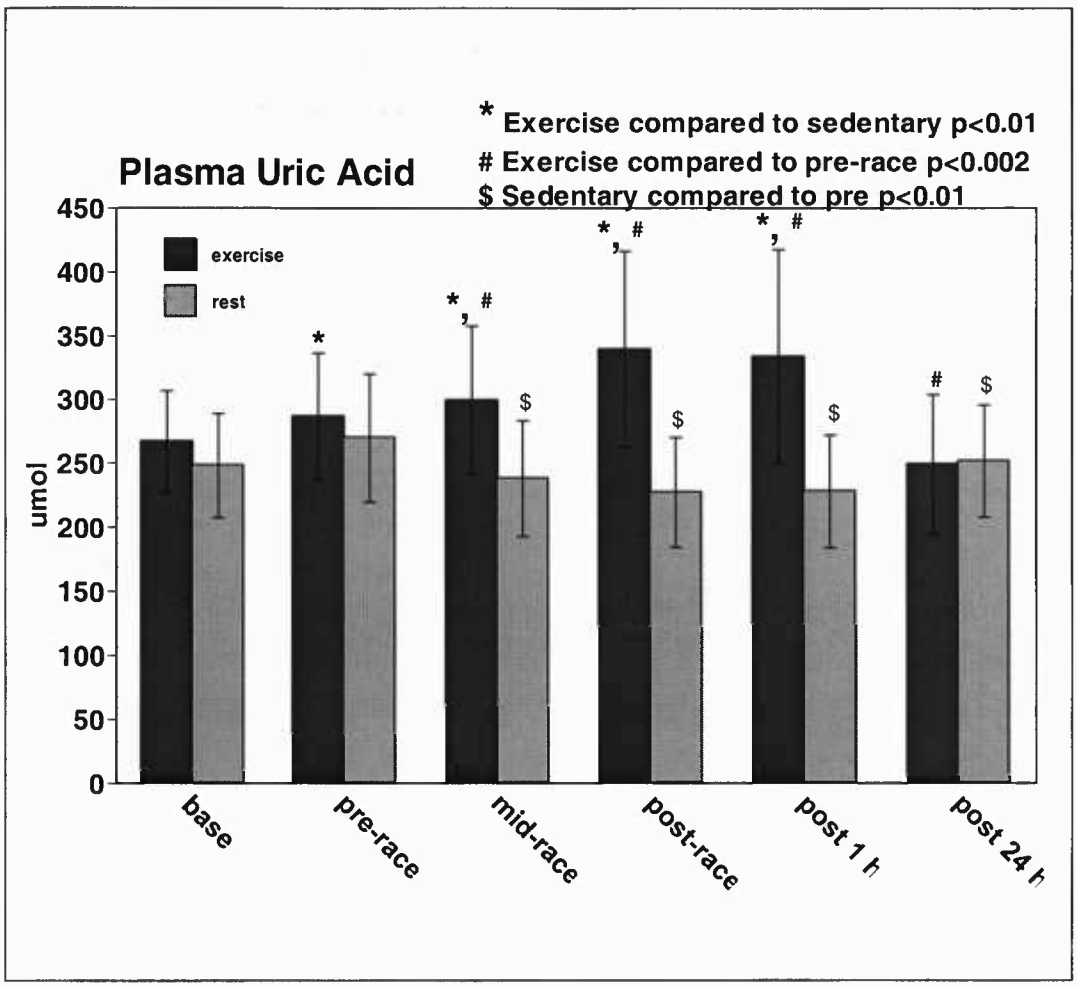
3.4.5. Ascorbic Acid

Endurance exercise is thought to increase oxidative stress and since ascorbic acid is the most effective water-soluble antioxidant in the plasma (45), we hypothesized that ascorbic acid levels would decrease in response to exercise. During the race trial, plasma levels of ascorbic acid *increased* from 75 ± 11 μM 30 minutes prior to the race to 117 ± 16 μM by mid-race ($p<0.0001$), 158 ± 36 μM at race end ($p<0.0001$) and remained elevated one hour post-race (142 ± 37 μM $p<0.0001$) (Figure 14). These increases can be attributed in part to the 406 ± 169 mg vitamin C consumed by subjects during the event. Ascorbic acid levels fell dramatically, to well below pre-race levels 24 hours post-race (40 ± 5 μM $p<0.0001$). A decrease in plasma ascorbic acid levels 24 hours after exercise has been reported by others (12, 17, 47, 81), and may be attributed to the replenishment of tissue stores depleted during the exercise bout. A decrease below baseline levels indicates that vitamin C levels may have actually been depleted by the exercise *despite* the observed increase in plasma ascorbic acid levels *during* exercise.

Similar to the race trial, plasma ascorbic acid levels increased during the sedentary trial, also in response to the consumption of vitamin C supplements in the form of ergogenic aids. During the sedentary trial, plasma levels of ascorbic acid increased from 64 ± 10 μM 30 minutes prior to the sedentary period to 90 ± 16 μM at sedentary period end ($p<0.0001$) and remained elevated one hour post- sedentary period (89 ± 13 μM, $p<0.0001$) (Figure 14). The post-24 hour sample for the sedentary trial was lost.

Ascorbic acid levels were elevated at all time points except baseline during the race trial compared to the sedentary trial ($p<0.0001$) (Figure 14). This increase in ascorbic acid levels in response to exercise is consistent with earlier studies and

Figure 15. Uric Acid



is thought to be a result of the mobilization of ascorbic acid from the adrenal gland in response to cortisol (81, 82). All differences calculated for plasma ascorbic acid levels remained significant after adjusting data for changes in plasma volume except the difference at pre-race.

3.4.6. Uric acid

Uric acid, another water-soluble plasma antioxidant (31), was predicted to decrease in response to endurance exercise. In fact, uric acid followed the same

pattern as ascorbic acid during the exercise trial, although the changes were much less dramatic. As seen in (Figure 15), race trial plasma levels of uric acid increased from 286 ± 50 μM 30 minutes prior to the race to 339 ± 77 μM by race end ($p<0.0001$) and remained elevated one hour post-race (333 ± 84 μM, $p<0.0001$). Uric acid levels fell to below pre-race levels 24 hours post-race (249 ± 55 μM, $p<0.002$).

Plasma levels of uric acid followed a much different pattern during the sedentary trial. Levels decreased during the sedentary trial from 270 ± 50 μM 30 minutes prior to the sedentary period to 238 ± 45 μM by mid- sedentary period ($p<0.0001$), 227 ± 43 μM at sedentary period end ($p<0.0001$) and remained depressed one hour post-sedentary period (227 ± 44 μM $p<0.0001$) (Figure 15). Levels did not return to pre-trial levels by 24 hours post-sedentary period (251 ± 44 μM $p<0.009$). These minor changes in concentrations are probably not physiologically relevant.

Uric acid levels were elevated at all time points during the race trial compared to the sedentary trial ($p<0.004$) except at baseline and 24 hours post-race (Figure 15), lending support to the hypothesis that exercise causes increased purine metabolism and subsequent formation of uric acid (83).

3.5. DISCUSSION

The most important finding of this study was the increased rate of vitamin E turnover during endurance exercise compared to the sedentary period. This is the first time that reactive oxygen species have been reported to increase the rate of vitamin E disappearance, a finding that indicates increased lipid peroxidation during exercise. This hypothesis is confirmed by our findings that plasma F_2-isoprostane levels increased significantly during the 50 km ultramarathon, but not

during the corresponding sedentary trial. The argument that this increase was due to artifact is diminished by the fact that increases in isoprostanes remained significant after correcting for changes in plasma volume. F_2-isoprostanes are a reliable measure of in vivo lipid peroxidation as they are chemically stable, specific end-products of arachidonic acid oxidation (84). During oxidative stress, arachidonic acid is increasingly susceptible to peroxidation and, importantly, F_2-isoprostanes are elevated in the plasma of subjects with known oxidative stress, such as smokers and patients suffering from diabetes, cystic fibrosis and Alzheimer's diseases (61-63, 85). The observed increase in F_2-isoprostanes in our ultramarathon study supports our hypothesis that the runners experienced increased lipid peroxidation.

Vitamin E (α–tocopherol) is a potent peroxyl scavenger and acts to protect polyunsaturated fatty acids (PUFA) within phospholipids of biological membranes against lipid peroxidation (6). We observed an increase in total plasma vitamin E, consistent with the findings of other investigators studying the response of plasma vitamin E to ultra-endurance exercise (47, 69). This increase was likely the result of a shift in the interorgan distribution of vitamin E (86), rather than a response to the consumption of vitamin E supplemented ergogenic aids since d_0 α-tocopherol increased during the exercise trial, but not the sedentary trial. Another possibility is that the increased levels of d_3-α-tocopherol during the sedentary trial were due to the upregulation of the α-tocopherol transfer protein. Although this mechanism has not been well elucidated, a recent study by Tamai et al (43) reported upregulation of the transfer protein in response to increased oxidative stress associated with diabetes.

A surprising observation in this study was that 12 hours after consuming the capsule, plasma d_3-labeled *RRR*- α–tocopherol levels were higher during the race

trial than the sedentary trial Figure 12. There are a number of explanations for this incongruous result. The variation in plasma response between the two trials may be due to differences in the rate of absorption. Absorption may have been influenced by the high level of anticipation prior to the ultramarathon run, but not the sedentary trial. It is reasonable to believe that subjects were agitated prior to the 50 km race, which could have delayed absorption of the labeled α–tocopherol. Assuming that they were more relaxed the night before the sedentary trial, differences in the time frame of absorption could have effected the shape of the absorption curve and/or a shift in the curve. In future, it would be beneficial to increase the number of blood draws in the time immediately following consumption of the capsules in order to be able to calculate area under the curve and thus account for skewed or shifted curves. Importantly, in some subjects the sedentary protocol d_3-α–tocopherol concentrations were higher than the race d_3-α–tocopherol concentrations, yet the plasma disappearance rates from the plasma were still faster during the race than the sedentary trial.

Congruous with the recent standards set by the Institute of Medicine (38) we observed a 2:1 ratio of natural to synthetic • • tocopherol twenty-four hours after consumption of the capsule containing deuterated tocopherols. It is worth mentioning that there were trivial amounts of deuterated tocopherols present in the plasma at baseline, this is because deuterium is a naturally occurring, stable isotope. According to the CRC Handbook of Chemistry and Physics (87), there is one atom deuterium present per 6000 ordinary hydrogen atoms. The amounts observed in our study were so minute, as to be inconsequential. Importantly, deuterium labeled • • tocopherol had returned to trivial levels by the baseline time

point of the sedentary trial, confirming that one month was a long enough wash-out period between trials.

In order to account for changes in lipoprotein secretion and normalize the data for inter-subject variability in cholesterol levels, d_3 labeled α-tocopherol per cholesterol (d_3/cholesterol) ratios were calculated. After correcting for d_3/cholesterol, differences at the 24 hour time point were eliminated implying that those differences were due to changes in lipoprotein secretion. Despite consuming similar foods, triglycerides and cholesterol data suggest higher lipoprotein secretion during exercise. Therefore, the faster vitamin E disappearance could be due to a faster lipoprotein turnover rather than a faster oxidation rate. By expressing the data as deuterated vitamin E/cholesterol, some of the variance due to lipoprotein turnover should be eliminated. However, the importance of lipoprotein turnover can not be ignored.

The finding that vitamin E turnover was faster during the race is most striking considering that the water soluble antioxidants ascorbic acid and uric acid both increased during the race trial. One possible explanation for this result is that ascorbic acid does not play a role in the prevention of lipid oxidation or that lipid peroxidation was occurring in a separate compartment from the ascorbic acid. A more plausible explanation is that because subjects' pre-race plasma ascorbic acid levels (75.2 ±11.2 μM) were already well above the concentration required to saturate tissues ≥ 50 μM (42, 88), consumption of vitamin C during the race did not have an effect on lipid peroxidation (88). In this case, elevated plasma ascorbic acid levels may have masked the effects of ascorbic acid quenching lipid peroxides. In future studies it will be important to recruit subjects that have plasma levels < 50

μM or control the diet long enough to achieve plasma levels < 50 μM, in order to maximize the likelihood of seeing an effect on lipid peroxidation.

Considering the high intakes of vitamin C in the form of carbohydrate drinks and pastes during the race (and sedentary period), it is not surprising that plasma ascorbic acid levels increased during both trials. The finding that plasma ascorbic acid levels increased to a greater extent during the race than during the sedentary period is consistent with the findings of others (16, 17, 47, 81) who reported increased plasma ascorbic acid levels in response to exercise. In addition to the high dietary intakes of vitamin C, the 48% increase in plasma ascorbic acid may be due in part to efflux from the adrenal gland in response to cortisol (81, 82) and/or the release of ascorbic acid from other tissue sites such as leukocytes or erythrocytes (16). Lending credence to the former theory, Gleeson et al (81) have demonstrated a positive correlation between the increase in cortisol and the increase in plasma ascorbic acid levels in response to exercise.

The 56 % higher plasma levels of ascorbic acid during the race compared to the sedentary trial may not be entirely accounted for by the theory of a redistribution of tissue ascorbic acid. The majority of vitamin C intake for all subjects was in the form of a carbohydrate powder that was premixed with water before the race. For the sedentary trial, subjects were given the powder to mix themselves and many subjects later reported having eaten the powder rather than diluting it with water. Since vitamin C absorption is both saturable and dose-dependent (18) it is plausible that subjects eating the carbohydrate powder without diluting it with water absorbed less vitamin C than when they drank it over time during the race.

Uric acid is an end product of purine metabolism and has been suggested to function as an antioxidant (1). Our observation that plasma uric acid increased in response to exercise is consistent with the findings of others (12, 47, 89). The exercise-induced increase in plasma uric acid may be due to enhanced purine oxidation in muscle (12, 47, 89). It may also be due to an increase in the activity of the adenylate cyclase system (4, 12). During intensive exercise such as the present ultramarathon race, there is increased likelihood that the enzyme responsible for the conversion of xanthine to uric acid will be xanthine oxidase. As discussed previously, the enzyme xanthine oxidase generates the superoxide radical as a by-product (83) and thus may contribute to oxidative stress during exercise.

Combined, results of the present study including increased plasma ascorbic acid, α-tocopherol, and uric acid, reflect an enhanced oxidative defense system in response to ultraendurance exercise. These findings lend support to previous studies reporting increased antioxidant enzymes (13) and antioxidant nutrients(10, 16, 47) in response to extreme exercise.

Although all of the statistical differences we calculated for this data remained significant after correcting the data for changes in plasma volume, we chose to report the uncorrected data. One reason for this is that there is a great deal of controversy over the use of hematocrit/hemoglobin for the determination of percent plasma volume changes. Schmidt et al (90) have argued that the hematocrit/hemoglobin method leads to an overestimation of change in plasma volume due to intravascular hemolysis. In contrast, Johansen et al (91) reported that the use of the hematocrit/hemoglobin method leads to an underestimation of change in plasma volume by as much as 50% during antiorthostatic maneuvers such as running, when compared to plasma volume changes determined by a direct tracer-

dilution method. Therefore, in future studies, it will be important to use direct tracer-dilution methods such as the Evans blue dye dilution technique. Finally, it is important to consider that it is the actual concentration of the parameter that the body reacts to, regardless of changes in plasma volume. This is most evident in the case of F_2-isoprostanes, which have biological activity of their own, acting as potent vasoconstrictors and/or causing platelet aggregation (58).

Levels of water-soluble molecules that are filtered by the kidney such as vitamin C, uric acid, urea and vitamin B_6 metabolites (76) increased during the race. Based on parameters measured, it is not possible to determine if these increases were due to their increased production or tissue release, decreased excretion or both. The question arises as to whether or not these and other parameters that increased during the exercise trial, such as F_2-isoprostanes, were real changes or if they reflected an impairment in renal function. It should not be overlooked that one of the physiological functions of F_2-isoprostanes is that they are potent renal vasoconstrictors (58). The possibility exists that increased oxidative stress resulted in elevated production of F_2-isoprostanes and therefore decreased renal function is a result of oxidative stress.

4. Conclusions

My hypothesis that exercise increases oxidative stress was supported by the findings that the rate of vitamin E turnover during endurance exercise was faster compared to the sedentary period and that plasma levels of free F_2-isoprostanes increased significantly during the 50 km ultramarathon, but not during the corresponding sedentary trial. Although these findings suggest an increased requirement for antioxidants in athletes, they are too preliminary to be able to make a recommendation as to whether or not endurance athletes should consume antioxidant supplements.

By taking advantage of the most recently developed techniques for assessing lipid peroxidation, i.e. vitamin E biokinetics, F_2-Isoprostanes, and tracking circulating antioxidants, the proposed study has helped us answer the question of whether extreme endurance exercise results in oxidative stress with much greater certainty than previous studies.

Future studies are underway in an attempt to repeat these findings in a treadmill run trial carried out under the controlled conditions of the laboratory. This will allow us to eliminate some of the confounding variables of the present study such as UV light exposure and muscle damage associated with downhill running. Another important study will be to repeat the present study with the addition of long-term supplementation with antioxidants prior to the exercise bout and the incorporation of a double-blind placebo controlled design.

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